cAMP Response Element-binding Protein Monomers Cooperatively Assemble to Form Dimers on DNA

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We have analyzed the properties of cAMP response element-binding protein (CREB) in solution with emphasis on dimerization and effects of phosphorylation. Using a purified CREB fusion protein, a novel dye-label technique, and sedimentation equilibrium analysis, we directly and conclusively demonstrate that, unlike Jun and Fos, CREB dimerization is DNA-dependent. CREB exists primarily as a monomer in solution and cooperatively assembles on DNA to form dimers. Sedimentation equilibrium analysis also indicates that dimerization is unaffected by cAMP-dependent protein kinase-phosphorylation or by the symmetry of the cAMP-responsive element binding site. Filter binding assays reveal that CREB binding is unaffected by phosphorylation regardless of the symmetry of the cAMP-responsive element binding site. Our results suggest that structurally similar members of the same bZIP superfamily may differ significantly in their regulation at the level of dimerization.

The cAMP response element-binding protein (CREB) regulates genes that control diverse biological functions such as memory (1), gluconeogenesis (2), opioid tolerance (3), spermatogenesis (4), and circadian rhythms (5). CREB regulates genes via binding to the cAMP-responsive element (CRE) (6, 7). In addition, CREB activity is modulated by phosphorylation at one or more sites within a kinase-inducible domain (8–11). Like most members of the bZIP superfamily, CREB has a modular structure consisting of distinct and separable DNA binding, dimerization, and transactivation regions (12). The kinase-inducible domain, which harbors a cluster of consensus phosphorylation sites for various protein kinases, lies within the two glutamine-rich transactivation domains (Q1 and Q2) toward the amino terminus of CREB (12, 13). The DNA binding (basic) and dimerization (leucine zipper) domains are adjacent to each other and occupy the carboxyl terminus of the protein (12).

While many protein kinases are able to modify CREB within its kinase-inducible subdomain, the effects of phosphorylation are not thoroughly understood. Most information on the effects of CREB phosphorylation has been obtained using cAMP-dependent protein kinase (PKA), which modifies a single serine site at position 133 (8, 9). Phosphorylation at Ser133 is known to increase the association of a 265-kDa nuclear protein, CREB-binding protein (CBP), and the homologous P300 (14, 15). Phosphorylation by PKA does not appear to alter the global conformation of CREB (16). Rather, recent structural studies support a model by which phosphorylation promotes direct electrostatic interaction between CREB and CBP/P300 (17, 18). The binding of CBP cannot, however, account for all of the effects of CREB phosphorylation. For instance, CREB can also be modified by calmodulin kinase II at positions 133 and 142 (10). However, a CREB mutant that mimics the calmodulin kinase II-phosphorylated protein is unable to support transcriptional induction under these conditions although CBP is still able to associate with CREB (19). Phosphorylation at positions 133 and 142 may have effects other than abrogation of CBP binding. It is possible that CREB phosphorylation influences properties of CREB such as binding affinity or dimerization.

It is generally accepted that bZIP proteins form dimers on DNA based on many in vitro and in vivo studies of yeast GCN4 and mammalian CREB/ATF and Jun/Fos families of transcription factors (20–25). However, it is not clear whether CREB is always self-associated as a dimer or whether the dimerization is always necessary for DNA binding and transcriptional regulation. For example, the CREB monomer has been reported to be the “active” form (26). The DNA binding affinity of some ATF family members is known to increase via dimerization upon association with the human T-cell leukemia virus trans-activator TAX protein, suggesting that CREB may not always exist as a dimer in solution (27, 28). There has also been considerable disagreement as to whether phosphorylation of CREB influences its binding affinity or dimerization. In vitro, no differences in DNA binding of purified CREB have been observed upon PKA phosphorylation in gel shift assays (29, 30). However, in the presence of cAMP, the DNA binding activity of CREB is known to increase, particularly at nonsymmetric CREs (31, 32).

Previously, the dimerization state of CREB has been inferred from use of truncated partners (33) and gel electrophoresis techniques (29, 30) in which the gel matrix has the potential to form or to disrupt DNA-protein complexes. However, solution kinetic and spectroscopic studies have suggested that CREB dimerization may be DNA-dependent (34). If this is the case, then binding isoforms that have assumed dimer binding to the CRE may be incorrect. If and under what conditions the full-length CREB transits between monomer and dimer remains uncertain. Further, how phosphorylation influences dimerization and binding by various kinases is largely unknown.

To clarify the effects of phosphorylation on the dimerization potential of CREB, we have undertaken a systematic study of...
dimerization, binding, and transcriptional effects supported by CREB. In our studies, we employ exclusively solution techniques to avoid any potential artifacts produced by gel electrophoresis of CREB-DNA complexes. In this report, we focus on dimerization and binding properties of CREB before and after phosphorylation by PKA. The effects of PKA on the structure and function of CREB are well established, so there is no confusion concerning the site or extent of modification. In our experiments, we directly measure the dimerization state under solution conditions using sedimentation equilibrium analysis (35) including a novel dye-label technique and a purified CREB fusion protein. We demonstrate that, unlike Jun and Fos, CREB dimerization is completely DNA-dependent. CREB exists primarily as a monomer in solution and cooperatively assembles on DNA to form dimers. CREB binding as well as dimerization is unaffected by PKA-induced phosphorylation. Our results suggest a model in which structurally similar members of the same bZIP superfamily differ significantly in their regulation at the level of dimerization.

**MATERIALS AND METHODS**

**Expression and Purification of His<sub>6</sub>-CREB**

The His<sub>6</sub>-CREB fusion protein was constructed by inserting the entire rat CREB-1 cDNA (9) together with a histidine tag into the pET22b expression vector (Novagen, Madison, WI). The histidine tag, comprising six consecutive histidines (His<sub>6</sub>), was located at the 5′ terminus of the coding region. BL21(DE3) cells (Novagen, Madison, WI) were transformed with the plasmid containing the fusion proteins, and expression of the protein was induced by treatment with 600 μM isopropyl-1-thio-β-D-galactopyranoside. Bacterial cells were harvested and lysed by lysozyme followed by sonication in binding buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol, pH 7.8). The cell extract was clarified by centrifugation and then directly applied to metal-chelated affinity resin (Novagen, Madison, WI) charged with Ni<sup>2+</sup>. The bound His<sub>6</sub>-CREB was eluted by a stepwise increase in imidazole from 50 to 500 mM. Purified His<sub>6</sub>-CREB, which eluted at 200 mM imidazole, was concentrated under N<sub>2</sub> by an ultrafiltration apparatus (Amicon, Beverly, MA) with a 30-kDa molecular mass cut-off filter. The concentrated protein was dialyzed with 20 mM Hepes, 100 mM KCl, 10 mM dithiothreitol (DTT), pH 7.5.

**Phosphorylation of His<sub>6</sub>-CREB**

Phosphorylation of His<sub>6</sub>-CREB utilized the catalytic subunit of PKA (Promega, Madison, WI). A ratio of 1 unit of enzyme/5 pmol of protein was employed in each phosphorylation reaction. Samples were incubated with PKA at 30 °C for 15 min in 20 mM Hepes buffer (pH 7.5), 100 mM KCl, 1 mM Mg<sup>2+</sup>, and 100 μM dithiothreitol. The sample was incubated with 1.0–2.5 μg calf thymus phosphatase as described previously (20). The phosphorylated CREB (at Ser 133) as described previously (20). The extent of phosphorylation was determined by adding the distance from the middle of each fringe to the axis of the rotor (o) (40). Utilizing the interferometric optical system, the interferogram was acquired on technical pan film (Eastman Kodak Co.). The distance (r) from the middle of each fringe to the axis of the centrifuge was determined by adding the distance from the middle of each fringe to the wire of the cell under the microcomparator and the distance from the meniscus to the axis of the rotor (a) (41). The absorbance data using the nonlinear Levenberg-Marquardt fitting routine (41) according to the equation,

\[
A(r) = A_o e^{-a o/r} + \frac{1}{r} + \frac{1}{r^2}
\]

where A is the absorbance, A<sub>o</sub> is the absorbance at the meniscus, a is the angular radius, o is the partial specific volume of the component, and M is the molecular weight of the component, which is obtained from the fitting. Both optical systems yielded similar results.

**Analysis of DNA-Protein Complexes by Fluorescein End Labeling of DNA**—For DNA-protein complexes, His<sub>6</sub>-CREB and P-His<sub>6</sub>-CREB were incubated with calf thymus phosphatase (Promega, Madison, WI) for 10 min at 37 °C. The His<sub>6</sub>-CREB or P-His<sub>6</sub>-CREB were loaded into the sample sector, and 215 μl of dialysate was added into the reference sector of each cell. At room temperature, equilibrium was achieved at 24 h when the rotor speed was 12,000 rpm. The partial specific volume (v<sub>p</sub>) of His<sub>6</sub>-CREB was calculated to be 0.72 ml/g based on its amino acid sequence and the partial specific volume of each amino acid (v<sub>c</sub>) (42). Utilizing the interferometric optical system, the protein boundaries were monitored at 258 nm, the wavelength that gives maximal difference in absorbance between the sample and the reference. Molecular weights for one or two components were obtained from the best fit of the data using the nonlinear Levenberg-Marquardt fitting routine (41) according to the equation,

\[
A(r) = A_o e^{-a o/r} + \frac{1}{r} + \frac{1}{r^2}
\]

where A is the absorbance, A<sub>o</sub> is the absorbance at the meniscus, a is the angular radius, v<sub>p</sub> is the partial specific volume of the component, and M is the molecular weight of the component, which is obtained from the fitting. Both optical systems yielded similar results.

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inverting DNA effective density (ρ) in salt solution (43). Since average DNA ρ is about 1.7 g/ml (44), ρ of the DNA is 0.588 ml/g (ρ = 1.0). The partial specific volumes of protein-DNA complexes were estimated by the equation,

\[ \nu = \nu_p + \nu_c \]  (Eq. 2)

where \( \nu_p \) and \( \nu_c \) are partial specific volume of protein (\( \rho \)) and oligonucleotides (\( \rho \)) and \( \nu_p \) and \( \nu_c \) represent the weight fraction of CREB and oligonucleotides in the complex. The \( \nu_c \) of the DNA-protein complex was 0.695 ml/g for a His6-CREB dimer and 0.679 ml/g for a His6-CREB monomer. For cooperativity studies, the molar ratio of protein to DNA ranged from 0 to 2.0. When subsaturating amounts of protein were used, fitting was performed assuming a two-component system. Between ratios of 0 and 0.8, one of the components was fixed to be the free DNA.

**Gel Filtration**

Purified His6-CREB (6 ml) was applied to a 2.5 × 110-cm Sephacryl S-200 HR column that had been packed and equilibrated with Hepes buffer (20 mM Hepes, 100 mM KCl, 10 mM DTT, pH 7.5). The column was calibrated by blue dextran 2,000 and protein molecular mass standards between 13.7 and 2,500 kDa (Amersham Pharmacia Biotech). Void volume (\( \nu_c \)) of the column was 203 ml. Proteins were eluted at a flow rate of 1.5 ml/min and collected as 4-ml aliquots. Fractions were concentrated 40-fold by a Centricon-30 concentrator (Amicon, Beverly, MA) at 4 °C and were analyzed by antibody detection using a rabbit anti-CREB Ab as described under “Antibody Detection of His6-CREB and P-His6-CREB.”

**Limited Proteolysis and Peptide Sequencing**

The His6-CREB-DNA complexes were formed by incubation of 1.1 nmol of His6-CREB or P-His6-CREB with 2.4 nmol of 27-bp somatostatin oligonucleotide at room temperature for 1 h. For limited proteolysis reaction, His6-CREB and P-His6-CREB or their DNA complexes were incubated with trypsin at a weight ratio of 100:1 (protein:trypsin). Since PKA (which is also a substrate incubated with trypsin at a weight ratio of 100:1 (protein:trypsin) was added to the CREB samples. However, ATP was not included in these reactions to prevent phosphorylation. The reactions were incubated at 37 °C and terminated by the addition of SDS sample buffer, and proteolytic fragments were separated on 15% SDS-PAGE gel. For amino acid sequencing, proteolytic products were transferred by electroblotting from SDS-PAGE separating gel to polyvinylidene difluoride membrane (Millipore, Bedford, MA) in transfer buffer (190 mM glycine, 25 mM Tris, 15% methanol). After transfer, bands on the membrane were visualized by staining with Coomassie Brilliant Blue solution (0.1% Coomassie Brilliant Blue R250, 40% methanol, 1% glacial acetic acid). Each proteolysis band was excised and sequenced by Edman degradation (pulsed liquid method) in a protein sequencer (Applied Biosystems 492 Precise Protein Sequencing System, Perkin-Elmer).

**RESULTS**

**His6-CREB Fusion Protein Functions Normally**—The His6-CREB is purified almost to homogeneity in 200 mM imidazole elution from an Ni\(^{2+}\)-charged affinity column (Fig. 1A). Because the histidine tag adds 33 amino acids to native CREB-1, the purified fusion protein migrates more slowly on SDS-PAGE compared with native CREB (Fig. 1B). That the protein band is His6-CREB is confirmed by antibody detection (Fig. 1B) and by its ability to specifically bind to CRE-containing oligonucleotides (Fig. 1C). Further, the His6-CREB fusion protein was efficiently phosphorylated by PKA (Fig. 1, D and E). In previous studies, we had found that the DNA-protein complex containing phospho-CREB migrates more slowly than CREB under gel shift conditions. After modification by PKA, the entire His6-CREB-shifted band migrates more slowly relative to complexes of unmodified His6-CREB (Fig. 1D). We confirmed that greater than 95% of the His6-CREB is modified by PKA phosphorylation using isoelectric focusing (data not shown) and incorporation of radiolabeled ATP. All of the modified CREB is detected by an antibody that recognizes only a Ser\(^{133}\)-phosphorylated form of CREB (20) and a regular anti-CREB-1 antibody (Fig. 1E). Taken together, these data suggest that our preparation of His6-CREB and P-His6-CREB represents a pure population of each form, suitable for physical studies.

To ensure that His6-CREB functions normally, we tested its binding ability to CRE and non-CRE-containing oligonucleotides (Fig. 1C). The His6-CREB binds specifically to the oligonucleotides containing CRE sites but not to the non-CRE sites in gel shift assays (Fig. 1C). Of most importance, the His6-CREB fusion protein functions normally in vitro (Fig. 2A). Transient transfection of His6-CREB and with the somatostatin-CAT reporter plasmids reveals that His6-CREB is equal in efficiency to native CREB in promoting transcription of somatostatin (Fig. 2A). Further, His6-CREB supports stimulated transcription after phosphorylation with equal efficiency to that of native CREB (Fig. 2A). The CAT activity of expressed His6-CREB is indistinguishable from native CREB whether or not the CREB is phosphorylated by PKA. We conclude that histidine tail causes no unexpected alterations in form or function of CREB. Below we will refer to His6-CREB as CREB and to the...
The Apparent Binding Constant of CREB Is Not Changed by PKA-dependent Phosphorylation—To quantify the effects of phosphorylation on the binding affinity of CREB in solution, we measured the dissociation constants of CREB and P-CREB by filter binding. We observed no change in the apparent $K_D$ among the different templates containing both symmetric and asymmetric CREs (compare Som CRE and Dyn CRE-1; Fig. 2B). Binding is, however, CRE-dependent, since the dissociation constant is small for CRE-containing oligonucleotides (Som CRE and Dyn CRE-1; Fig. 2B) and larger for non-CRE containing template (Enk AC; Fig. 2B) or for a point mutation (Dyn CRE-1.2; Fig. 2B) that abrogates CREB binding to dynorphin CRE-1 in gels (38). For P-CREB, the binding constant is indistinguishable from CREB for any template examined (Fig. 2B). We conclude that the apparent binding constant of CREB is not changed by PKA-dependent phosphorylation regardless of the symmetry of the CRE region.

Dimerization of CREB Is DNA-dependent—The apparent dissociation constants were calculated based upon an assumption that a preformed CREB dimer binds to the CRE in both unphosphorylated and phosphorylated forms. Many nonsolution studies using truncated or mutant molecules and gel shift analysis indicate that dimers of unphosphorylated CREB bind to a CRE (33, 45). However, whether CREB remains as a dimer after phosphorylation has not been directly examined. If phosphorylation by PKA alters the self-association state of CREB, then the binding constants will be affected. To determine the form of CREB both on and off DNA in solution, we measured the self-association state of CREB and P-CREB by sedimentation equilibrium analysis. The advantage of the method is that the molecular mass of a macromolecule or macromolecular complex can be directly measured in solution. The concentration of CREB in PC-12 cells has been estimated to be roughly 0.4 $\mu M$ (46). Based on this estimate, concentration conditions used for the experiment were selected to be near physiological, ranging between 0.5 and 16.0 $\mu M$.

We found that both CREB and P-CREB free in solution are primarily monomers in a concentration range from 0.5 to 16.0 $\mu M$ (Fig. 3A). The predicted molecular mass of CREB from rat is 36.6 kDa (9), and the addition of the histidine tag increases the molecular mass to 40.4 kDa. The best fit to the concentration gradient reveals a major form with a molecular mass very close to the predicted mass of a monomer (Fig. 3A). Some amount of dimer and aggregation were apparent in the sample. Since the analysis yields a “weight average” molecular mass, the presence of some aggregated material skews the fitted value toward a slightly higher mass. Despite the error, it is clear from the data that CREB is not a dimer (80.8 kDa) under near-physiologic concentrations. Gel filtration chromatography confirms the results of the sedimentation experiment. The major eluate co-migrated with the 43-kDa protein standard, indicating that the monomer was the predominant form (Fig. 3A). Some amount of dimer and aggregation were apparent in the sample.

We also detected a small amount of dimer (Fig. 3B) and some high molecular weight aggregation (not shown). The profile of free CREB was similar to that of free P-CREB (not shown). Thus, PKA-mediated phosphorylation does not promote dimerization of the free protein.

We found, however, that CREB dimerization is CRE-dependent. To monitor CREB-DNA complexes in solution, we labeled DNA templates with fluorescein (Fig. 4A). Labeling with fluorescein allows detection of only the DNA-bound CREB complex as long as the molar ratio of CREB is high enough to saturate the DNA. Free CREB does not absorb at 494 nm (the $\lambda_{max}$ absorbance of fluorescein), and under saturating conditions, there is no detectable level of free DNA (Fig. 4A). As templates,
we utilized a canonical, symmetric somatostatin CRE (5'-TGACGTCA-3') (47) or an asymmetric CRE3 from the rat prodynorphin gene (dynorphin CRE3) (38). In solution, both somatostatin CRE and dynorphin CRE3 have similar binding affinities for CREB (Fig. 2B) and both bind CREB as dimers (Fig. 4B). Further, there is no significant difference in the bound complex if CREB is phosphorylated (Fig. 4B). We conclude that neither the binding constant nor CREB dimerization is significantly altered by PKA-dependent phosphorylation. However, we show that CREB monomers must assemble on DNA to form dimers. Unlike Jun-Fos or Jun-Jun complexes, then, CREB dimerization is DNA-dependent.

Consistent with DNA-dependent dimerization, we find distinct differences in the trypsin sensitivity within the C terminus of CREB after DNA binding (Fig. 5, A and B). Limited proteolysis of CREB and P-CREB, free in solution (Fig. 5A) or bound to DNA (Fig. 5B) yielded prominent bands based on their migration on the gel (Fig. 5; represented as bands 1–5). Each band was purified and sequenced to determine the site of cleavage (Fig. 5C). Compared with the monomeric free CREB (Fig. 5A), the C-terminal portion of the molecule is protected in the presence of DNA (Fig. 5B). The greatest effects are the protection of cleavage at positions 284 and 292 (leading to loss of bands 2 and 3) and the accumulation of a resistant band 1 (Fig. 5C). Protection of cleavage at 284 and 292 is expected due to PKA phosphorylation that is independent of DNA binding (Fig. 5). The greatest effects are the protection of cleavage at positions 284 and 292 (leading to loss of bands 2 and 3) and the accumulation of a resistant band 1 (Fig. 5C). Protection of cleavage at 284 and 292 is expected after DNA binding, since these sites are within the DNA binding domain (Fig. 5C). However, band 1 contains the dimerization domain, which is not in contact with the DNA surface (48). Despite the presence of several trypsin cleavage sites, protection from proteolysis within this region (in either bound CREB or P-CREB) occurs only after DNA binding (Fig. 5, A and B). These data are consistent with DNA-dependent CREB dimerization. We also observe subtle differences in cleavage intensity due to PKA phosphorylation that is independent of DNA bind-
ing. The band 5 doublet, cleaved from Arg 136 and very close to
the phosphorylation site Ser133, appears delayed after phospho-
rylation. Also, the intensity of the band 5 doublets are reversed
after phosphorylation by PKA. These results indicate that PKA
induces subtle conformational change in CREB near the phos-
phorylation site and that dimerization occurs on DNA.

Dimerization of CREB at the CRE Site Is Cooperative—There
are two possible mechanisms by which CREB monomers can
assemble on a DNA template. Each has biological significance.
Monomers of CREB may assemble cooperatively on DNA to
form dimers. In this case, a fraction of CRE binding sites may
be unoccupied while others bind dimers. In a cooperative
model, little to no CREB monomer should be bound at any ratio
of CREB to DNA (Fig. 6 A). Alternatively, CREB monomers
may bind in a noncooperative manner, filling CRE sites as
monomers before CREB dimers are formed (Fig. 6 A). In the
noncooperative model, a CREB monomer has equal probability
to bind to a free CRE site or to a CREB monomer-occupied site.
Therefore, at a low protein:DNA molar ratio, the major form in
solution should be a monomer-DNA complex. However, with
the increase of the ratio of protein to DNA, we expect that the
fraction of the dimer-DNA complex should increase (Fig. 6 A).

We have directly tested these two models by measuring the
CREB self-association state on bound DNA templates as a
function of increasing protein concentration. We found that
CREB binds cooperatively to CRE templates. CREB complexes
were preformed with defined input ratios of CREB over a wide
range, from 0 to 2.0. Gradients from sedimentation equilibrium
experiments were fit using two components, one of which was
free DNA. The best fit of the data to Equation 1 reveals a
molecular weight consistent with the presence of a dimer
bound to DNA independent of the ratio (Fig. 6 B).

We conclude that binding of monomeric CREB and P-CREB to both
symmetric and asymmetric CRE sites is cooperative. The PKA-de-
pendent phosphorylation has no apparent effect on CREB
dimerization at the CRE sites.

**DISCUSSION**

Many studies have led to the understanding that CREB is a
dimer when bound to DNA (20, 33, 45). However, how CREB
dimerizes or the effect of phosphorylation on the dimerization
state has not been examined in solution. CREB binding has
been analyzed largely using gel shift assays and other nonsol-
ution techniques that cannot reliably distinguish between
dimer and monomer states. Here we directly measure the effect of
phosphorylation on CREB dimerization in solution. Our re-
ults reveal several novel properties of CREB dimerization and
clarify aspects of DNA binding that have been previously
reported.

First, free CREB does not homodimerize. Although CREB
has never been crystallized, it has been suggested that dimer-
ization of CREB is like that of Jun and Fos based on structural similarities of these bZIP family members (12, 49). Both solution (50) and x-ray diffraction (21) studies have shown that Jun and Fos dimerize on or off DNA as Jun-Jun or Jun-Fos complexes (21, 50). However, we find that, unlike Jun-Fos or Jun-Jun complexes, CREB is largely a monomer in solution. CREB indeed dimerizes at a CRE binding site but dimerization is DNA-dependent. Detailed spectroscopic and kinetic studies of bZIP peptides had suggested that CREB might bind to DNA as a monomer (34). Our results for the first time directly demonstrate this is the case for native CREB. Further, monomeric forms of bZIP family members may play a significant role in transcriptional activation. For example, SKN-1, a Caenorhabditis elegans transcriptional factor, binds DNA with high affinity as a monomer through its basic region, which is very similar to that of bZIP proteins (51, 52). Thus, dimerization may not be required for some bZIP proteins to either bind DNA or activate transcription. Structurally similar members of the bZIP family are very different with respect to dimerization.

The rules that govern dimerization are not fully understood. Many studies have correlated structural features of interfaces among leucine zipper proteins with their dimerization potential. Association between the two zippers is primarily due to van der Waals forces between the hydrophobic residues at the a and d positions in the helix (12, 53). Because of the structure and size, the presence of leucine at the d positions allows most stable packing at the dimer interface (54) and promotes formation of coiled-coils with a dimer of parallel helices. The hydrophobic residues a are much less conserved than positions d, although alanine substitutions at some a positions can be destabilizing (23). Additionally, the interface of the dimer is reinforced by interhelical salt bridges between polar residues at positions e and g (12). Dimerization is weakened if there is electrostatic repulsion between these positions. The electrostatic force between the interhelical e and g positions has been used to explain why Fos does not homodimerize (55, 56). CREB has a typical leucine zipper region in which the d positions are occupied exclusively by leucine and the a positions are generally occupied by valine or leucine. Overall, the e and g residues in CREB-1 are nearly neutral (12). Based on structural features of its leucine zipper interface, the lack of a significant CREB homodimer in solution is difficult to explain. However, residues outside the leucine zipper region also contribute to dimer stability (56–58), and the CREB protein may contain residues beyond the leucine zipper region that are important for dimer destabilization. Whatever the constraints, DNA binding clearly leads to dimerization. It remains to be determined whether bound CREB dimer forms a canonical leucine zipper interface or whether monomer binding simply facilitates the association of a second molecule.

Second, our results directly demonstrate that the binding constant of CREB does not need correction for alterations in its “form”. Several groups have previously reported that PKA does not influence the apparent binding constant of CREB. However, in all other studies, dimerization after CREB phosphorylation was assumed but not tested. Here, we directly show that CREB cooperatively assembles on DNA to form a dimer and that dimerization is unaffected by PKA-dependent phosphorylation. Using nuclear or cellular extracts, PKA has been reported to increase the binding affinity of CREB particularly at nonsymmetric CRE sites (32). Based on our results, an increase in CREB binding under these conditions is likely to involve other dimerization partners. In this regard, it is significant that CREB binds cooperatively to the CRE. Cooperative binding allows greater regulation of transcription, since the monomer can receive signals that may influence the choice of dimer partner, which, in turn, attracts adapter proteins such as CBP. It has been reported that CREB can dimerize with ATF-1 (59) and CREM (60). Phosphorylation may control selection of a CREB dimerization partner. We have recently found that, in contrast to PKA, calmodulin kinase II phosphorylation abrogates CREB-dependent transcriptional activity and CREB dimerization on the prodynorphin CRE.2 Our results suggest that CREB phosphorylation at positions 133 and 142 by calmodulin kinase II may promote the association of a “repressive” dimer partner. Alternatively, CBP may not bind to the monomer if phosphorylation at 133 and 142 inhibits the ability of CREB to dimerize.

Finally, our studies suggest that dimerization properties may distinguish the roles of structurally similar bZIP proteins. Our results show that free CREB exists primarily as a monomer in the cell but cooperatively binds to DNA as a dimer. Neither DNA binding activity nor the self-association state of both free and DNA-bound CREB are regulated by PKA-induced phosphorylation regardless of the symmetry of the CRE site. In contrast, Jun and Fos proteins readily dimerize in solution and bind to DNA as a dimer. CREB is a constitutive protein in the cell, while expression of Jun and Fos are induced in response to external stimuli. Jun and Fos dimerize for immediate function but are not always present in the cell. Thus, bZIP proteins of the CREB family are constitutively transcribed but are regulated at the level of dimerization. In contrast, Jun and Fos are regulated at the level of transcription but are designed to constitutively dimerize for immediate function. Structurally similar members of the same superfamily appear to differ significantly in their regulation at the level of dimerization.

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