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Engineered leucine zippers show that hemiphosphorylated CREB complexes are transcriptionally active

(transcription factor dimerization/protein kinase A/cyclic AMP response element-binding protein)

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ABSTRACT **The ability of basic/leucine zipper transcription factors to form homo- and heterodimers potentially increases the diversity of signaling pathways that can impinge upon a single genetic element. The capacity of these proteins to dimerize in various combinations complicates the analysis of their functional properties, however. To simplify the functional analysis of CREB dimers, we mutated selected residues within the leucine zipper region to generate proteins that could only heterodimerize. These mutants allowed us to determine whether phosphorylation of both CREB subunits was necessary for transcriptional activation. Our results reveal that hemiphosphorylated CREB dimers are half as active as fully phosphorylated dimers. It is possible, therefore, that the degree of phosphorylation of CREB complexes could modulate the transcriptional responses of specific genes to cAMP.**

Transcription factors typically contain distinct DNA-binding and activation domains. In many cases, the biological activities of these factors depend on their ability to dimerize, and the various classes of transcriptional activators frequently contain discrete subdomains that participate in this process. The utility of dimerization is twofold: in addition to increasing the specificity of DNA binding, this process increases the diversity of protein complexes that can interact with a single genetic element. This diversity is likely to be important for integrating information from distinct signal transduction pathways. Our studies were directed toward understanding the functional properties of heterodimeric complexes that bind to the cAMP-regulated enhancer (the cAMP response element, CRE; refs. 1 and 2).

Although several CRE-binding proteins have been described (3), most studies have focused on CREB (4, 5), which is believed to mediate transcriptional signals directed by the cAMP and calcium second-messenger pathways (6–8). Within its activator domain, CREB contains consensus phosphorylation sites for several protein kinases. The most critical of these sites is serine-133, which can be phosphorylated by both protein kinase A (PKA) and calcium/calmodulin-dependent kinase II (8, 9). Mutation of this serine to alanine destroys the ability of CREB to mediate transcriptional activation (9).

CREB homo- and heterodimerizes through its leucine zipper, an amphipathic α-helix containing a heptad repeat of leucine residues at the hydrophobic dimerization interface (10). Leucines or other nonpolar residues occupy most of the **a** and **d** positions of the interface (Fig. 1). As in other basic/leucine zipper (bZIP) proteins, conserved polar residues within this region of CREB probably affect zipper alignment and stability, while neighboring charged residues affect dimerization specificity. The asparagine residue that occupies the **a3** position in the leucine zipper of many bZIP proteins is predicted to destabilize the dimer complex (11). Charged residues in the **g** position of one helix generally form complementary or repulsive electrostatic interactions with those in the **e** position of the adjacent helix (11). These electrostatic interactions have been shown to be important determinants of the dimerization potential of proteins in the bZIP class (12).

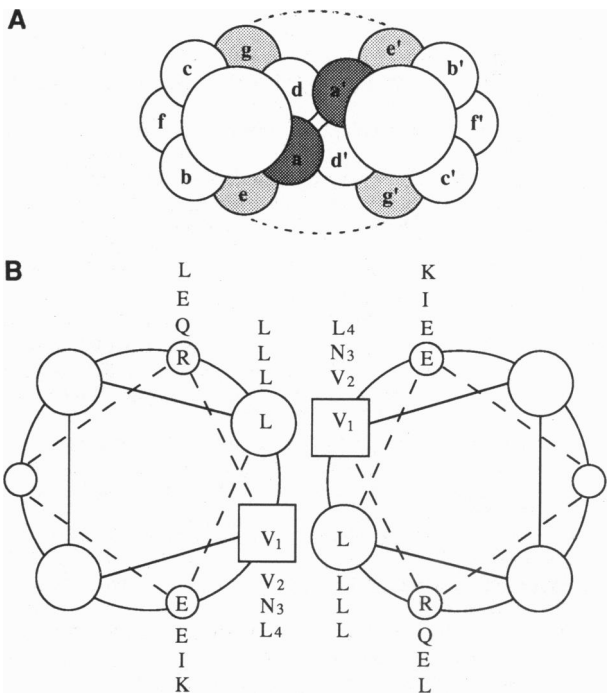

FIG. 1. End view of parallel α-helices in a dimerized leucine zipper. (*A*) Residues in the **a** and **d** positions form the hydrophobic dimerization interface. Electrostatic interactions (dashed lines) occur between charged residues in the **e** and **g** positions. (*B*) Helical-wheel representation of the CREB leucine zipper. Substitutions were made for the asparagine residue (N3) within the dimerization interface and for selected residues in the **e** and **g** positions.

In vitro studies have shown that the various CRE-binding proteins heterodimerize only in specific combinations (13). Although it is predicted that the formation of such heterodimers generates complexes with novel binding or transcriptional activities (14, 15), there are relatively few well-documented examples of this mode of regulation in intact cells. For example, CREB heterodimerizes with related

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Abbreviations: CRE, cAMP-responsive element; CAT, chloramphenicol acetyltransferase; PKA, protein kinase A; bZIP, basic/leucine zipper.

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proteins such as ATF-1 and CREM (16, 17), but the functional properties of these dimer combinations are unknown. It is unclear, therefore, whether the negatively acting CREM transcription factors block transcriptional activation by forming nonfunctional heterodimers with the positive activators CREB and ATF-1 or by forming homodimers that prevent the activators from binding to the CRE. The former model provides a mechanism that should maximize the efficiency and specificity of the negative regulators, whereas the latter mechanism, while less efficient, could additionally impinge upon genes that are regulated by proteins that do not dimerize with CREM, such as ATF-2.

A major problem in the functional analysis of bZIP transcription factors is their propensity to associate in both homodimeric and heterodimeric complexes. For example, a mixture of two proteins in this class can generate three distinct dimer combinations—two homodimer forms and one heterodimer. We addressed this problem by designing CREB molecules that could form only heterodimers. These molecules could then be used to determine the functional properties of specific heterodimeric transcription factor complexes. As a test of this strategy, we asked whether PKA-mediated transcriptional activity required that both subunits in the CREB dimer were phosphorylated. Surprisingly, we found that CREB complexes phosphorylated on only one subunit were precisely half as active as complexes containing two intact PKA phosphorylation sites.

MATERIALS AND METHODS

Plasmid Construction and Expression. A CREB 327 cDNA was isolated from a λgt11 human hypothalamic library and was subcloned into the *Hin*dIII/*Xba* I site of pALTER (Promega) for *in vitro* mutagenesis. Mutagenesis was carried out as described in protocol for the Altered Sites *in vitro* mutagenesis system (Promega). *Kpn* I–*Sma* I restriction fragments encoding the DNA-binding domain and the mutant leucine zipper region of CREB were then subcloned into a pET-11d expression plasmid (Novagen) containing a full-length CREB 327 insert (provided by T. Usui, Vollum Institute). A pET-11d plasmid containing a truncated form of wild-type or mutant CREB (designated the short form) was also constructed to allow visualization of the protein heterodimers in gel mobility-shift assays. These constructions, which encoded a protein with a 60-amino acid deletion, were generated by removing a *Pvu* II–*Stu* I restriction fragment that included nucleotides 351–531. pET-11d plasmids were resequenced by using T7 DNA polymerase (Sequenase, United States Biochemical) to confirm that each contained the correct mutations. The full-length CREB 341 expression vector used for *in vivo* transfection experiments was produced by cloning a rat CREB cDNA (obtained from M. Montminy, Salk Institute) into the expression vector pRL/RSV (Invitrogen). Mutations were introduced into this vector by replacing the CREB 341 *Kpn* I/*Sma* I fragment (encoding the DNA-binding domain/leucine zipper region) with the *Kpn* I–*Sma* I mutant fragments generated in the CREB 327 cDNA. A form of CREB 341 that could not be phosphorylated by PKA (9) was generated by mutating serine-133 to alanine. The plasmids RSV-luciferase (18) and RSV-PKA (19), in which expression is directed by the Rous sarcoma virus promoter, were obtained from M. Wilkinson and R. Maurer, respectively (Oregon Health Sciences University). The plasmid SS-CAT, containing sequences of the rat somatostatin gene from −71 to +53 placed 5′ to the coding region of the chloramphenicol acetyltransferase (CAT) gene, was constructed previously (1).

Bacterial Expression of Recombinant CREB Proteins. pET-11d plasmids encoding wild-type, mutant, and CREB short forms were transformed into the BL21(DE3) strain of *Escherichia coli*. Bacterial cultures were grown to an OD_{600} of 0.6 in Luria broth containing ampicillin (100 μg/ml). Cells were induced to express recombinant proteins by the addition of isopropyl β-D-thiogalactopyranoside (0.5 mM). After 3 hr, 7.5 ml of cells were harvested and lysed by sonication in 300 μl of a solution containing 50 mM dithiothreitol and 50 mM EDTA in phosphate-buffered saline. Bacterial debris was cleared by centrifugation after the addition of 30 μl of 10% (vol/vol) Triton X-100. The cleared supernatant was heated to 72°C for 2 min, and the precipitated proteins were again cleared by centrifugation. The supernatant was used in gel mobility-shift assays. The concentration of recombinant CREB in each extract was determined by densitometric analysis of samples electrophoresed in SDS/polyacrylamide gels stained with Fast Stain (Zoion Research, Allston, MA).

Gel Mobility-Shift Assays. Mixtures of extracts were heated to 72°C, added to a 20-μl reaction buffer [10 mM Tris, pH 7.5/50 mM NaCl/10% (vol/vol) glycerol/5 mM MgCl/1 mM dithiothreitol/1 mM EDTA containing 1 μg of poly(dI-dC) and 3.9 μg of bovine serum albumin], and incubated at 37°C with 15 fmol of end-labeled somatostatin CRE oligonucleotide. Reaction mixtures were loaded onto a nondenaturing 6% polyacrylamide gel and were electrophoresed at 200 V for 10 hr. Gels were dried and autoradiographed with Kodak XAR film.

Molecular Modeling. Mutant and wild-type CREB leucine zippers were modeled into the GCN4 leucine zipper by using a Silicon Graphics work station and MIDASPLUS software (27). The coordinates were kindly provided by T. Alber, University of Utah.

Cell Culture and Transfection Assays. F9 mouse teratocarcinoma cells (provided by M. Montminy, Salk Institute) were grown on gelatin-coated plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells (3.5×10^5) were plated on 10-cm dishes 18 hr before transfection. DNA was transfected by using the Transfinity calcium phosphate transfection system (BRL) and plasmids in the following amounts: 5 μg of SS-CAT, 2 μg of RSV-luciferase, and the indicated amounts of RSV-PKA and RSV wild-type and mutant CREBs. The total DNA in each transfection was 20 μg, the balance consisting of pRC-RSV. Fresh medium was added 24 hr after transfection and cells were collected for assay 24 hr later. CAT activity was determined by the method of Seed and Sheen (20) and was normalized to luciferase activity (18) as a control for transfection efficiency.

RESULTS

The **a3** asparagine of CREB and related proteins is thought to function by destabilizing the hydrophobic interface of the bZIP dimer (11). Thus, we initially targeted this particular site for mutation. We reasoned that this position could be made even more destabilizing in the homodimer form if it was substituted with a residue with a formal positive or negative charge. By site-directed mutagenesis, the wild-type asparagine was replaced with either aspartic acid, lysine, arginine, or histidine. The aspartic acid substitution was made to introduce a formal negative charge, and the lysine and arginine mutations were made to introduce formal positive charges. The histidine mutation introduced an amino acid that could be charged or uncharged, depending upon the pH. We proposed that destabilization of the mutant homodimers could occur by charge repulsion or by steric hindrance. We additionally hypothesized that the asparagine/histidine heterodimer could be stabilized by a hydrogen bond between the histidine and the asparagine and by van der Waals contacts with residues surrounding the **a3** positions.

Recombinant CREB proteins were expressed in bacteria and purified by heating. This procedure removes most of the bacterial proteins, which precipitate at 72°C. Recombinant

CREB is extremely heat-stable and returns to its native form, as analyzed by circular dichroism, after cooling to room temperature (Jane Richards, R.G.B., and R.H.G., unpublished work). Samples of each extract were electrophoresed in SDS/polyacrylamide gels to assess the quantity and purity of recombinant protein in each extract. Recombinant CREB represented >90% of the protein in each preparation (data not shown), and differences in protein expression among individual extracts were <10%.

The ability of the recombinant proteins to dimerize *in vitro* was analyzed by gel mobility-shift assays. In these assays, full-length proteins were mixed with a truncated form of CREB, 60 amino acids shorter, to allow visualization of heterodimer complexes. Except for the aspartic acid substitution, each of the mutations decreased the amount of homodimer and increased the amount of heterodimer formed (data not shown). The greatest effect was seen after mutating the **a3** position to histidine, but proteins containing this substitution still did not completely prevent formation of homodimers containing the wild-type asparagine.

To further decrease homodimerization of CREB molecules containing the **a3** asparagine, we additionally mutated amino acids in the **e** and **g** positions (Fig. 2*A*). The resultant CREB proteins, designated pZIP3 and pZIP12, combined only as heterodimers when equimolar amounts of the full-length and deleted forms were mixed (Fig. 2*B*). In mixtures containing a large excess of pZIP3, a small amount of homodimer was also seen. This form presumably resulted from retention of the wild-type **a3** asparagine. No homodimerization was detected in mutant proteins that contained a histidine residue in the **a3** position.

The biological activities of the mutated proteins were assessed by transient transfection assays in F9 teratocarcinoma cells. These cells were chosen because they have low levels of functional CREB and PKA (9). Cells were transfected with a somatostatin–CAT reporter gene and plasmids encoding PKA, RSV-luciferase (to control for transfection efficiency), and various amounts of CREB. Reporter activity depended on the amount of wild-type CREB expression vector used and, in each instance, increased after addition of PKA (Fig. 3). Activity of the CREB form containing the pZIP12 leucine zipper (341_{ZIP12}) was negligible, even in the presence of PKA, at all amounts tested. The slightly higher activity of 341_{ZIP3} is consistent with the results of the binding studies and indicates that homodimerization of the mutant proteins can be forced to occur at high expression levels if the complementary CREB mutant is omitted. When the complementary CREB mutants were combined, transcriptional activities of the mixtures were indistinguishable from those of wild-type CREB. To minimize background activity from the homodimer forms, 1.5 μg of each CREB plasmid was used in subsequent studies.

The leucine zipper mutants were next used to determine the biological activities of CREB dimers that could be phosphorylated on only a single subunit. For these studies, serine-133 in each of the CREB leucine zipper mutants was replaced with alanine. This mutation, designated M1, has been used previously to establish the role of PKA phosphorylation in CREB activation (9). In the absence of PKA, M1-mutated CREB containing a wild-type leucine zipper has the same activity as native CREB (Fig. 4; see also ref. 21), suggesting that the serine-to-alanine substitution does not alter general aspects of CREB structure. The same level of PKA-independent activity was directed by wild-type/M1 heterodimers (data not shown). PKA slightly decreased the transcriptional activity of the M1 homodimers containing either wild-type or mutated leucine zippers. This apparent decrease in activity is due, at least in part, to the low level of PKA stimulation of the RSV-luciferase plasmid that is used for normalization. The activities of heterodimers that con-

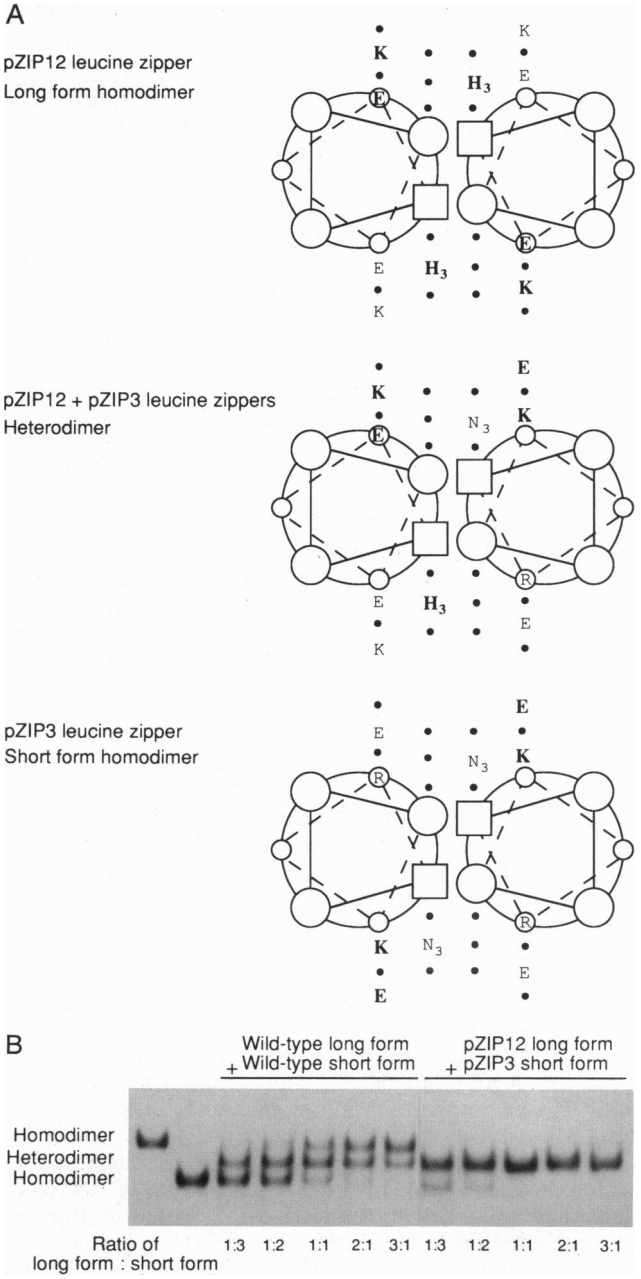

FIG. 2. Binding properties of mutant CREB proteins. (*A*) Helical-wheel diagrams showing the mutated amino acid residues (bold letters). In the pZIP12 leucine zipper homodimer (*Top*), a negatively charged glutamate (E) replaces the arginine (R) at the **g1** position, and a lysine (K) replaces the glutamate at the **g3** position. These mutations should result in charge–charge repulsions in the homodimer forms. This homodimer should also be destabilized by the histidine (H) substitution at the **a3** position. In pZIP3 (*Bottom*), a lysine substitution at the **e2** position and a glutamate substitution at the **e4** position destabilize the homodimer form. pZIP3/pZIP12 heterodimers (*Middle*) regenerate the favorable electrostatic interactions of the wild-type proteins and contain an additional stabilizing asparagine (N)–histidine configuration at the **a3** position. Thus, this combination should be favored over either homodimer form. (*B*) Gel mobility-shift assays. To visualize the homodimer and heterodimer complexes, a 60-amino acid fragment was deleted from the activator domains of the wild-type CREB and pZIP3 isoforms. Proteins were mixed in the ratios shown and analyzed in nondenaturing 6% polyacrylamide gels. When equal amounts of the full-length and deleted wild-type CREB proteins were mixed, homodimers and heterodimers formed in a 1:2:1 ratio. In contrast, the mutated CREB proteins combined only as heterodimers when mixed in equimolar concentrations.

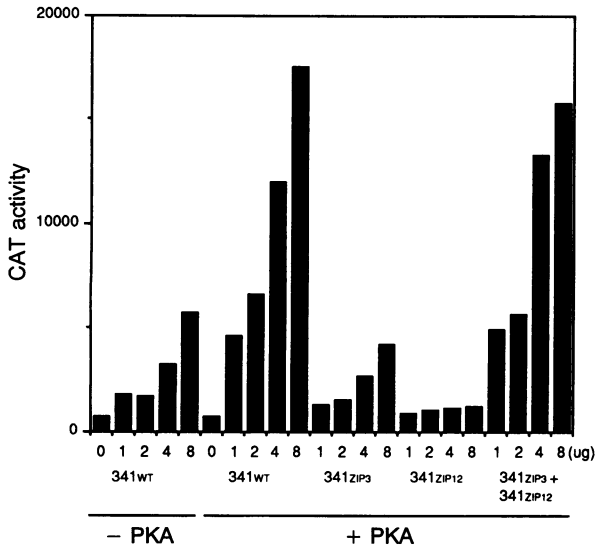

FIG. 3. Biological activities of CREB leucine zipper mutants. CREB 341 plasmids containing wild-type, ZIP3, or ZIP12 leucine zippers were introduced into F9 teratocarcinoma cells along with 5 μg of somatostatin–CAT reporter and 5 μg of PKA expression vector as indicated. CAT activity is normalized to luciferase expression. Numbers refer to the total amount of CREB plasmid (0, 1, 2, 4 or 8 μg) in each transfection. Mixtures of CREB vectors containing the complementary leucine zipper mutations reconstitute wild-type activity.

tained only a single intact PKA phosphorylation site were precisely half as high as complexes containing two phosphorylation sites. Thus, transcriptional activation mediated by

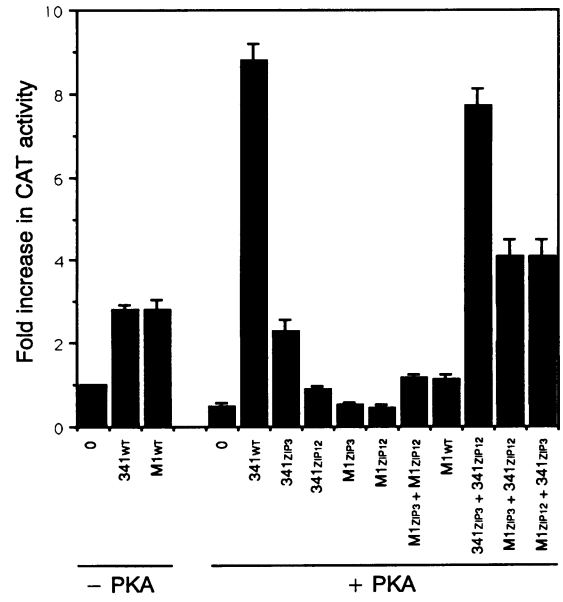

FIG. 4. Transcriptional activity of hemiphosphorylated CREB complexes. F9 cells were transiently transfected with a somatostatin–CAT reporter and a mixture of CREB expression vectors. 341 and M1 refer to CREB isoforms containing either a wild-type serine or mutant alanine at the PKA site within the activator domain. Subscripts WT, ZIP3, and ZIP12 refer to the wild-type or mutant leucine zipper domains. A total of 3 μg of CREB expression vector was used in all experiments except those denoted 0, in which the CREB vector was omitted. Experiments were performed in the absence or presence of 5 μg of PKA expression vector as indicated, and results were normalized to luciferase activity. Complexes containing only one intact PKA phosphorylation site were half as active as those containing two phosphorylation sites. Values (from five experiments) represent means ± SE.

PKA does not require phosphorylation of both subunits of the CREB dimer.

DISCUSSION

Our strategy for engineering the CREB leucine zipper domains was guided by several previously reported studies of related bZIP proteins. Two observations in particular suggested that substitutions at the **a3** position might alter the dimerization properties of these factors. (*i*) The amino acid that typically occupies this position is a unique polar residue in an otherwise hydrophobic dimerization interface (Fig. 1). An asparagine is conserved at this position in the leucine zippers of C/EBP-1, c-Jun, GCN4, and CREB, suggesting that this particular residue has an important function in dimerization specificity (11). By interrupting the continuity of the hydrophobic interface, this asparagine is thought to destabilize the leucine zipper and may thereby permit reversible dimerization. This process may be important for allowing factors to exchange partners under different physiological conditions. (*ii*) Although *in vivo* studies examining the dimerization properties of GCN4 leucine zipper mutants showed that the **a3** position was relatively tolerant of amino acid substitution, an aspartic acid substitution, in particular, was able to disrupt dimer formation (22). This finding suggested that charge repulsion at this position might be particularly effective in destabilizing homodimer formation. We proposed that mutations that introduce larger polar or charged residues at this position would further destabilize homodimer formation. At the same time, it was important to identify an amino acid that would allow dimerization with CREB molecules containing the wild-type **a3** asparagine.

Of the four mutations tested, the **a3** asparagine-to-histidine substitution was most effective in reducing homodimer formation. To elucidate how this mutation affected stability of the CREB dimers, we modeled the histidine substitution into the crystal structure of the GCN4 leucine zipper (11) by using a Silicon Graphics work station and MIDASPLUS molecular-modeling software. In the histidine homodimer, the best modeled histidine–histidine interaction is a hydrogen bond that is 3.3 Å long and nonplanar. It was not possible to maintain this hydrogen bond and still make favorable van der Waals contacts with neighboring residues, however. Thus, we predict that unfavorable hydrogen-bond geometry may destabilize homodimers containing the histidine mutation.

Molecular modeling also suggested the basis for the stability of the asparagine/histidine heterodimer. The asparagine–histidine contact can be modeled to have a 2.8-Å hydrogen bond between the carbonyl oxygen of asparagine and the histidine N^{ϵ}. This bond length is optimal and the proton donor and acceptor are nearly linear. The van der Waals contacts between the histidine N^{δ} and an isoleucine within the same zipper helix, as well as between the histidine C^{δ} and a nearby leucine on the opposite helix, are predicted to stabilize the heterodimer form.

Amino acid substitutions were also made in the **e** and **g** positions of the CREB leucine zipper. Stabilizing electrostatic interactions between the corresponding residues have been observed in the GCN4 crystal structure (11). In addition, repulsive electrostatic interactions between amino acids in these positions are thought to prevent homodimerization of c-Fos, and stabilizing interactions are thought to direct the preferential heterodimerization of c-Fos with c-Jun (12). Nicklin and Casari (23) have confirmed this model by showing that a single amino acid substitution in the **g1** position, which converts the repulsive electrostatic interaction between residues in the **e** and **g** positions to an attractive one, allows c-Fos to homodimerize. A strategy similar to the one reported in this manuscript was used recently to direct heterodimerization of Myc and Max proteins (24). In that

case, mutant proteins with reciprocally modified e and g residues in their leucine zippers were transformation-defective when transfected individually into mammalian cells, but transformation-competent when combined. In our studies, mutations in the e and g positions alone were not sufficient to completely prevent homodimer formation. Only the mutant leucine zipper pZIP12, which contained a histidine substitution at the a3 position, was completely inactive in binding and transcription assays. Thus, the combination of a3, e, and g substitutions was required to direct the formation of CREB heterodimers exclusively.

The results of our transfection studies have several general implications for second-messenger-regulated gene expression. First, they provide evidence that the process of dimerization in bZIP proteins contributes to determining the level of transcriptional activation by juxtaposing two activation domains. These conclusions differ from those of Oliviero and Struhl (25), who found that heterodimers containing a GCN4 activation domain had nearly equivalent transcriptional properties whether they contained one or two activation domains. In their system, the degree of activation depended primarily on the number of binding sites upstream from the promoter. The difference between our results and those of Oliviero and Struhl may reflect differences in the mechanisms of PKA-inducible and acidic activator transcription factors.

The observation that hemiphosphorylated CREB complexes maintain the ability to respond to PKA, albeit at a half-maximal level, supports the concept that heterodimerization may allow the targeting of distinct second-messenger-mediated signals to a single genetic element. If two phosphorylated CREB subunits were required for transcriptional responsiveness, heterodimers containing distinct subunits that respond to different second-messenger pathways would not be capable of activating transcription. The approach utilized here should be useful for determining the biological functions of some of these other heterodimer complexes. Finally, the distinct activities of unphosphorylated, hemiphosphorylated, and fully phosphorylated CREB complexes suggest that cAMP-directed transcriptional signals may be modulated according to the degree of CREB phosphorylation. Three possible mechanisms could generate CREB dimers that are hemiphosphorylated—a submaximal response to PKA or to protein phosphatase I (26) or heterodimerization of CREB with a transcription factor that does not participate in PKA signaling.

Although the model that hemiphosphorylated CREB complexes are half as active as fully phosphorylated dimers is appealing physiologically, there was no reason *a priori* to expect this result. Additionally, the mechanism for the half-maximal level of activity of the hemiphosphorylated CREB complexes is unknown. It is possible that the hemiphosphorylated complexes may associate less efficiently with other proteins that are required for transcriptional activation. For example, the binding of a coactivator or general transcription factor could be enhanced by the presence of a second phosphorylated CREB subunit. This mechanism would not necessarily lead to a doubling of the transcriptional activity, however. A second possibility is that complexes that are phosphorylated on a single subunit may function directionally. Because the sequence of the CREB binding domain is symmetrical, the hemiphosphorylated dimer can bind to the CRE in two different orientations. It is possible that only one of these orientations is functional. In this instance, phosphorylation of the second CREB subunit would eliminate the inherent polarity of the hemiphosphorylated complex.

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