DNA Binding and Phosphorylation Induce Conformational Alterations in the Kinase-inducible Domain of CREB

IMPLICATIONS FOR THE MECHANISM OF TRANSCRIPTION FUNCTION

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Cyclic AMP response element-binding protein (CREB) mediates activation of target gene transcription by protein kinase A (PKA) phosphorylation at serine 133. This is followed by recruitment of the coactivators CREB-binding protein (CBP) or p300. Conversely, the decline in expression during the attenuation phase is linked to CREB dephosphorylation by nuclear phosphatases. The CREB bZIP domain, which promotes dimerization and promoter binding, as well as the kinase-inducible domain (KID), which interacts with the KIX domain of CBP/p300, are both largely unstructured in solution and become more structured once bound to their respective ligands. In this study, we biochemically characterize DNA- and phosphorylation-induced conformational alterations in CREB that may play a role in its transcriptionally poised, activated state. We find that sequence-specific DNA binding of pCREB renders the protein resistant to serine 133 dephosphorylation by protein phosphatase 1. Paradoxically, CREB bound to DNA and chromatin is efficiently phosphorylated by PKA, indicating that the KID region exists in a different conformation depending on its phosphorylation state. Consistent with this observation, we find that phosphorylation of DNA-bound CREB promotes an alternate conformation characterized by an apparent increase in the size or asymmetry of the complex and a qualitative change in proteolytic sensitivity. Together, our data indicate that DNA binding promotes a global conformational change in CREB that alters the structure of KID. PKA phosphorylation of KID in the DNA-bound state induces a phosphatase-resistant conformation that may prolong transcriptional activity.

The cyclic AMP response element-binding protein (CREB) induces expression of many cellular genes in response to phosphorylation by several kinases including the cAMP-dependent protein kinase (PKA).

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2 The abbreviations used are: CREB, cyclic AMP response element-binding protein; bZIP, basic leucine zipper; CBP, CREB-binding protein; C/EBP, CAAT/enhancer-binding protein; CRE, cAMP response element; EMSA, electrophoretic mobility shift assay; KID, kinase-inducible domain; NE, nuclear extract; OA, okadaic acid; pCREB, phosphorylated cyclic AMP-binding protein; pKID, phosphorylated kinase inducible domain; PKA, cAMP-dependent kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GST, glutathione S-transferase; FL, full-length; ds, double-stranded; DTT, dithiothreitol.

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been widely studied with variable results; some studies suggest an increased affinity of CREB for non-consensus CREs upon phosphorylation whereas others show no effect (18, 26–29).

It is well established that PKA phosphorylation of CREB (pCREB) promotes the interaction of KID with the KIX domain of CBP/p300, and subsequent activation of target genes. However, it is not clear how CREB maintains its phosphorylated status in the presence of nuclear phosphatases. In this report, we demonstrate that pCREB bound to CRE DNA acquires a protein phosphatase 1 (PP1)-resistant structure while the unphosphorylated form attains a structure that remains accessible to PKA. Most interestingly, we find that phosphorylation induces a further conformational change in DNA-bound CREB. This is demonstrated by an increase in the size or asymmetry of the phosphorylated protein/DNA complex and by a qualitative change in the proteolytic digestion pattern. Deletion of basic amino acids in KID mimics the phosphorylation-induced conformational alteration, suggesting that intramolecular interactions promote a more compact structure in CREB that are disrupted upon phosphorylation. These data are unexpected, as previously published studies suggest that the only significant structural alterations in the otherwise unstructured protein are induced solely within the bZIP domain and KID upon binding to their respective ligands (13, 17, 18, 20, 22). Together, our data show that the KID region has the ability to adopt two unique, DNA-induced conformations that are differentially recognized by opposing regulators.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification*—The bacterial expression plasmids for CREB327, CREB341 (30), and CREB341Δ121–125 (23) were expressed in *Escherichia coli* Rosetta BL21(DE3)pLysS cells and purified by heat treatment followed by heparin-Sepharose and size-exclusion chromatography. All CREB proteins were purified to >98% homogeneity and were free of contaminating nucleic acid. The bZIP327–332 plasmid was expressed and purified as previously described (31). CREB was phosphorylated with the catalytic subunit of PKA (Sigma, cat. P-2645) as previously described (31). Mock phosphorylated CREB was expressed and purified as previously described (31). CEM nuclear extract was prepared as previously described (33).

*Preparation of Ser19−radiolabeled pCREB*—Purified CREB was phosphorylated using the catalytic subunit of PKA (Calbiochem, cat. 539486) in a reaction containing 1 pmol of CREB, 2 units of PKA, and 0.25 pmol of γ-32P]ATP (6 µCi/pmol specific activity). The reactions were further incubated with unlabeled ATP (100 µM) to drive the phosphorylation reaction to completion. PKA was heat-inactivated at 75 °C for 10 min.

*Dephosphorylation of pCREB by CEM Nuclear Extract*—[32P]CREB was incubated with CEM nuclear extract (50 µg, unless otherwise indicated) at 30 °C for 30 min. in a buffer containing 50 mM Tris-HCl, pH 7.9, 12 mM MgCl2, 100 mM NaCl, 2 mM DTT, and 1 mM EDTA. Control reactions were incubated with buffer alone, in the absence of nuclear extract. The reactions were terminated by the addition of SDS sample dyes and resolved by 12% SDS-PAGE followed by autoradiography.

*Phosphatase Assays*—Dephosphorylation of pCREB was performed using either protein phosphatase 1 (New England Biolabs, cat. P0754S) or protein phosphatase 2A (Calbiochem, cat. 530508). [32P]CREB/bZIP heterodimers were formed by heating equimolar amounts of protein (10.4 µM total protein) to 65 °C for 10 min then cooling on ice. [32P]CREB samples (4 pmol) were incubated with PP1 in a buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, and 1 mM MnCl2. Reactions with PP2A were carried out in a buffer containing 50 mM Tris-HCl pH 7.0, 1.4 mM 2-mercaptoethanol, 1 mM benzamidine, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. The amount of phosphatase used in each experiment varied based on the activity of the enzymes. Reactions were carried out for 15 or 30 min. at 30 °C, as indicated. In the phosphatase inhibition assay, okadaic acid was included in the reactions at concentrations of 12.5, 25, 50, 100, 200 nM. Reactions were terminated by the addition of SDS sample dyes and resolved by 12% SDS-PAGE followed by autoradiography.

*Immobilized Template Assay*—A 643-bp promoter fragment was PCR-amplified from a plasmid template which carries four reiterated copies of an off-consensus CRE (5′-TGACGACA-3′) from the human T-cell leukemia virus, type 1 promoter (4TxRE/G-less) (34). Primers were designed to amplify the sequence between 187 to +456 and generate a fragment carrying a biotin group at the 5′ end. The binding of the gel purified fragment to streptavidin-coupled magnetic beads (Dynal Biotech USA, cat. 112.06) was performed according to the manufacturer’s instruction. The subsequent binding of proteins was carried out as previously described (35), and as indicated in each figure.

*Immunoblotting*—CREB proteins were resolved by 12% SDS-PAGE, and immunoblots were performed with antiphosphoSer133 CREB or anti-CREB antibodies (Santa Cruz Biotechnol, Ser133 cat. sc-7879 and C-21 cat. sc-186, respectively). Proteins were visualized with horseradish peroxidase-labeled corresponding secondary antibodies and ECL + chemiluminescence (Amersham Biosciences, cat. RPNI-2132).

*Oligonucleotides*—The sequence of the double-stranded (ds) oligonucleotides (IDT) used in the indicated assays are as follows: Primers for PCR amplification of the CRE promoter (4TxRE/G-less) (34) top strand: 5′-Bio/CATCGATAAGCTT-
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CTAG-3’, bottom strand: 5’-CATGATACGCCAGGC-3’. Dephosphorylation protection assay, CRE oligo 1, top strand: 5’-AGTCAGCTCGCTTCAGGCGTACGTTGACGTAACCCCTCACCTCAGCTCAAAAAAC-3’. CRE oligo 2, top strand: 5’-AGTCAGCTCGCTTCAGGCGTACGTTGACGTAACCCCTCACCTCAGCTCAAAAAAC-3’. CRE oligo 3, top strand: 5’-AGTCAGCTCGCTTCAGGCGTACGTTGACGTAACCCCTCACCTCAGCTCAAAAAAC-3’. CRE oligo 4, top strand: 5’-AGTCAGCTCGCTTCAGGCGTACGTTGACGTAACCCCTCACCTCAGCTCAAAAAAC-3’. C/EBP oligo, top strand: 5’-AGTCAGCTCGCTTCAGGCGTACGTTGACGTAACCCCTCACCTCAGCTCAAAAAAC-3’.

Proteolysis—Protein samples were incubated with trypsin at a ratio of 1:100 (w/w, sequencing grade trypsin, Promega, cat. V5111). The reactions were performed with 3.8 μM CREB or pCREB, 38 μM KIX, and a 1.5-fold molar excess of CRE DNA (2.8 μM) (CRE oligo 1) relative to the dimer. The reactions containing KIX or DNA were preincubated for 30 min at 30 °C prior to the addition of trypsin. 4 μg of CREB was analyzed for each time point. Reactions were carried out at 25 °C in a buffer containing 50 mM ammonium carbonate, pH 7.9 and 10 mM DTT for the times indicated. Reactions were terminated with SDS sample dye and flash-freezing in liquid N2. Proteins were resolved by 10% Tris-Tricine SDS-PAGE and visualized by Comassie Brilliant Blue.

FoldIndex Analysis—FoldIndex is a simple tool to predict whether a given protein sequence is intrinsically unfolded. Folding predictions for CREB327 were plotted using the default window size of 51 amino acids and calculated by the Uversky method using average residue hydrophobicity and net charge (36, 37).

Accessibility of CREB Ser119 to PKA Phosphorylation—Free and DNA-bound CREB (8 pmol) were phosphorylated using 8 units of the catalytic subunit of PKA (Calbiochem, cat. 530508) and 2 pmol of [γ-32P]ATP (6 μCi/pm specific activity) as described above for the times indicated. The reactions were terminated using SDS sample dye and resolved by 12% SDS-PAGE. Reactions containing chromatin were resolved by 4–20% gradient SDS-PAGE. Gels were briefly stained and destained to visualize total CREB and histone proteins, followed by autoradiography.

Chromatin Assembly—The 643-bp CRE-containing promoter fragment (4TxE) was immobilized on magnetic beads as described above. The immobilized fragment was assembled into chromatin using Drosophila core histones, which were purified as previously described (38). Assembly reactions were performed as previously described (39, 40). Nucleosome assembly was confirmed by micrococcal nuclease analysis. Prior to performing the PKA assays, the assembled chromatin was washed thoroughly with 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA to remove free ATP and nucleosome assembly proteins.

Micrococcal Nuclease Assay—Micrococcal nuclease (MNase) digestion was performed on 2 μg of PCR-amplified CRE fragment (4TxE) assembled into chromatin, as previously described (41). Samples were resolved by 1.5% Tris borate agarose and visualized by SYBR® gold nucleic acid gel stain (Molecular Probes, cat. S-11949). GeneRuler™ 100 bp markers (Fermentas, cat. SM0323) were used as DNA size standards.

Electrophoretic Mobility Shift Assay—Protein-DNA complexes were formed by incubating a 26-bp 32P-end-labeled ds CRE oligonucleotide (0.5 nM) with the various CREB proteins (2.5 nM), as previously described (31). Heterodimers were formed by heating equimolar amounts of protein (10.4 μM total protein) to 65 °C for 10 min then cooling on ice. Samples were analyzed on a 5% (49:1 w/w acrylamide:bisacrylamide) non-denaturing gel in a buffer containing 40 mM Tris-HCl pH 8.5, 306 mM glycine and 0.1% Nonidet P-40, followed by autoradiography.

Gel Filtration Chromatography—CREB proteins used to form homodimers (7 μM) and heterodimers (3.5 μM each, 7 μM total) were heated to 65 °C for 10 min, placed on ice for 10 min, then incubated with a 21-bp CRE oligonucleotide (4.2 μM) for 1 h on ice in a 250-μl reaction. The protein/DNA complexes were injected onto a prepackaged analytical Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with buffer containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl2, 5 μM ZnSO4, and 2 mM DTT. The column was run at a flow rate of 0.5 ml/min, and protein/DNA absorbance was monitored at 280 nm. Blue dextran 2,000 and protein molecular mass standards between 14.4 and 669 kDa were used to calibrate the column. The void volume of the column was at ~7.8 ml. Elution volumes (Ve) of the complexes were normalized to the Ve of the DNA alone.

Image Processing and Data Reproducibility—Image Quant 5.1 software (Molecular Dynamics) was used to quantify band intensities. Background signal was subtracted from each band quantified. Images were processed in Adobe Photoshop enabling minor adjustments to brightness/contrast as needed (gamma was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint. All experiments presented in this manuscript were shown to be reproducible in at least three independent trials.

RESULTS

pCREB Is Dephosphorylated by Protein Phosphatase 1 Present in a T-cell Nuclear Extract—PKA phosphorylation of CREB at Ser133 leads to the recruitment of the coactivators CBP/p300 through specific interactions between the KID region of CREB and the highly conserved KIX domain of CBP/p300. This transcription factor-coactivator interaction promotes transcription of target genes. We were interested in determining the stability of the phosphorylated state of CREB in the presence of CEM nuclear extract in vitro, as this modification is critical to the physiological function of CREB. Previous studies have shown that CREB is dephosphorylated by protein phosphatases present in nuclear extracts from PC12 and HepG2 cell lines (10, 12).

To perform these studies, we used highly purified CREB327 that was PKA-phosphorylated at serine 119 (Ser119). This CREB isoform carries the full kinase-inducible domain (KID) with the consensus PKA phosphorylation site at Ser119 (versus Ser133 in CREB341) (42–44) and has similar transcriptional activity as CREB341 (45). CREB was phosphorylated by PKA and [γ-32P]ATP, incubated with increasing amounts of CEM nuclear extract, and analyzed by SDS-PAGE followed by auto-
radiography. Fig. 1A shows that the lowest amount of nuclear extract used in this experiment dephosphorylated CREB to near completion. The loss of signal was not caused by degradation of CREB, as proteolytic products were not observed (data not shown).

The protein phosphatases PP1 and PP2A are believed to account for 90% of the phosphatase activity in mammalian cells (46, 47). Both have been implicated in CREB dephosphorylation (10, 12, 13) and have been previously shown to be expressed in human T-cells (48). Okadaic acid (OA), a highly selective serine/threonine protein phosphatase inhibitor, is commonly used to identify the active protein phosphatases in a reaction based on the differential sensitivity of members of the protein phosphatase family to the inhibitor. PP2A has an IC$_{50}$ (50% inhibition concentration) between 0.1–1.0 nM whereas PP1 has an IC$_{50}$ between 10–100 nM (49). To identify the protein phosphatase(s) present in the CEM nuclear extract responsible for dephosphorylation, [32P]CREB was incubated with nuclear extract and increasing amounts of OA. Concentrations of OA that inhibit PP2A did not affect [32P]CREB dephosphorylation, however, we found that OA concentrations in the range that inhibit PP1 (100 and 200 nM) concomitantly inhibited [32P]CREB dephosphorylation by the nuclear extract (Fig. 1B). A graphical representation of the data obtained from three independent OA inhibition assays is shown in Fig. 1C.

Direct comparison of purified, recombinant PP1 and PP2A in a [32P]CREB dephosphorylation assay shows little or no effect of PP2A on dephosphorylation, whereas PP1 produced significant dephosphorylation of [32P]CREB (Fig. 1D). We did not observe [32P]CREB dephosphorylation under any reaction conditions or concentrations of PP2A (4–20 units per pmol pCREB), consistent with the results obtained using OA (data not shown). Together, these data suggest that PP1 is the primary protein phosphatase responsible for CREB dephosphorylation in CEM nuclear extracts, consistent with previously published data (10). PP2A may actually play a more indirect role in the CREB signaling pathway at a point upstream of CREB, i.e. in the dephosphorylation and inactivation of MAPKIV, one of the kinases responsible for CREB phosphorylation (50).

DNA Binding Inhibits CREB Dephosphorylation—We next examined whether DNA binding by pCREB influences dephosphorylation by nuclear extract using a PCR-generated fragment carrying four reiterated copies of a CRE cloned upstream of a minimal promoter (34). The upstream amplification primer carried a 5'-biotin group enabling conjugation of the fragment to streptavidin-coupled magnetic beads. For this experiment, unlabeled pCREB dimer was added in 2-fold molar excess relative to CRE binding sites, enabling analysis of CREB phosphorylation in both the unbound and DNA-bound fractions. Purified pCREB was preincubated with the immobilized template fragment, followed by incubation with nuclear extract. The extent of dephosphorylation was visualized by immunoblotting using an antibody directed against PKA-phosphorylated CREB. Surprisingly, we found that template-bound pCREB was highly resistant to dephosphorylation, whereas CREB free in solution was fully dephosphorylated (Fig. 2A, lanes 1 and 2, upper panel). A reprobe of the blot using an antibody that recognizes both phosphorylated and unphosphorylated CREB (CREB total, designated CREBt) confirmed that unbound pCREB was dephosphorylated by nuclear extract and was not lost because of proteolytic degradation (Fig. 2A, lane 2, lower panel).

To further explore the degree of DNA binding required for inhibition of CREB dephosphorylation, we performed an experiment in which increasing concentrations of a 43-bp CRE oligonucleotide were preincubated with purified [32P]CREB, followed by the addition of CEM nuclear extract. The results were analyzed by autoradiography (Fig. 2B). Molar equivalent concentrations of the CRE, relative to the pCREB dimer, were required for significant inhibition of CREB dephosphorylation. A graphical representation of the data obtained from three independent assays is shown in Fig. 2C. Together, these data indicate that when pCREB is bound to DNA, the phospho-Ser119 within the KID region is inaccessible to dephosphorylation by protein phosphatases.

**FIGURE 1. PKA-phosphorylated CREB is dephosphorylated by PP1 present in a T-cell nuclear extract.**

A. CEM nuclear extract dephosphorylates pCREB. [32P]CREB (25 nM) was incubated with the indicated amounts of CEM nuclear extract (NE) for 30 min and analyzed by SDS-PAGE and autoradiography. B. okadaic acid inhibits protein phosphatase 1 present in the CEM nuclear extract. [32P]CREB (25 nM) was incubated with nuclear extract for 30 min in the presence of increasing concentrations of okadaic acid (OA), as indicated. Samples were analyzed by SDS-PAGE and autoradiography. C. graphical representation of the effect of okadaic acid on CREB dephosphorylation. The percentage of dephosphorylated pCREB was calculated relative to the intensity of [32P]CREB in the absence of nuclear extract and okadaic acid, which was set to 0. The graph represents the results of three independent assays. D. protein phosphatase 1 dephosphorylates pCREB. [32P]CREB (25 nM) was incubated in the presence of CEM nuclear extract, PP1 or PP2A, for the times indicated. Samples were analyzed by SDS-PAGE and autoradiography.
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Inhibition of CREB Dephosphorylation Requires Sequence-specific DNA Binding—To determine whether DNA-induced resistance to dephosphorylation was sequence-specific, we analyzed the susceptibility of pCREB to dephosphorylation by PP1 in the presence of a variety of DNA elements. These elements included ds oligonucleotides carrying a consensus CRE (oligo 1), an off-consensus CRE (oligo 2), and a C/EBP site, each with identical flanking sequences. We also tested a promoter fragment carrying four off-consensus CREs, a randomized ds oligonucleotide, and the alternating copolymer, poly(dA-dT). pCREB dephosphorylation was measured in the presence of a 2-fold molar excess of each DNA element relative to the pCREB dimer, at a concentration above the Kd, to ensure complete binding (51). Binding reactions were analyzed by immunoblot using an antibody specific for PKA-phosphorylated CREB (Fig. 3A, upper panel). pCREB binding to all CRE-containing sequences protected the protein from PP1 dephosphorylation. The random DNA sequence, lacking a CRE, the C/EBP oligo and poly(dA-dT) were all ineffective at protecting pCREB from dephosphorylation. These data indicate that sequence-specific binding is required for pCREB protection from PP1 dephosphorylation. The CBP KIX domain (GST-KIX588–683) was included as a positive control, as KIX alone has previously been shown to protect CREB from dephosphorylation (13). An immunoblot showing total CREB protein in each reaction is shown (Fig. 3A, lower panel).

We next performed a time course assay with PP1 to directly compare the ability of DNA and KIX to protect CREB from dephosphorylation. We first established that PP1 remained active during the full 60 min time course (data not shown). [32P]CREB was preincubated with a CRE-containing ds oligonucleotide, KIX, or both molecules, then incubated with PP1 for the indicated lengths of time (Fig. 3B). As expected, [32P]CREB was rapidly dephosphorylated in the absence of DNA or KIX (Fig. 3B, lanes 1–5). We observed partial dephosphorylation of [32P]CREB in the presence of DNA over the length of the time course. [32P]CREB was fully protected from dephosphorylation in the presence of KIX (Fig. 3B). A graphical representation of the data obtained from three independent time course assays is shown in Fig. 3C.

Intramolecular Interactions Prevent pCREB Dephosphorylation When Bound to DNA—Based on these data, we addressed whether protection from PP1 dephosphorylation resulted from steric hindrance because of intermolecular contacts between the N-terminal, non-bZIP regions of the two CREB monomers bound to DNA. We compared dephosphorylation of full-length [32P]CREB homodimers to that of [32P]CREB:bZIP273–327 heterodimers in the absence or presence of DNA. Combining an equal molar ratio of [32P]CREB and bZIP results in the formation of three species: homodimer of full-length [32P]CREB, heterodimer of full-length [32P]CREB and bZIP, and homodimer of bZIP, which remains unlabelled. Reactions containing the full-length [32P]CREB (FL) homodimer or the [32P]CREB:bZIP (FL:bZIP) heterodimer were preincubated in the absence or presence of the CRE followed by treatment with PP1. Under these conditions, approximately half of the labeled [32P]CREB was present in the heterodimer complex. Therefore, all of the full-length [32P]CREB in the DNA-bound CREB:bZIP reaction should have been protected from dephosphorylation if intramolecular interactions surrounding pKID conferred protection. Consistent with this prediction, Fig. 3D shows that the full-length homodimer (FL) and the heterodimer (FL:bZIP) complexes were comparably protected from PP1 dephosphorylation in the presence of DNA at the 5 min time point. Therefore, DNA binding appears to induce a change in pCREB structure, resulting in resistance to PP1 enzymatic activity. This is conferred by intramolecular interactions within or near the pKID region. Consistent with this, basic residues of pKID have been shown to make supportive interactions with neighboring acidic regions within the same molecule (23).
**DNA Binding Inhibits CREB Dephosphorylation**

Limited proteolysis can be used to detect changes in conformation that may result in differences in band pattern or intensity. By comparing the trypsin cleavage pattern of pCREB in the absence and presence of the consensus CRE, we were able to further investigate the effect of DNA binding on CREB conformation. Conveniently, trypsin cleavage sites are primarily within the KID and bZIP regions of CREB, allowing visualization of any structural changes that specifically affect these domains (Fig. 4B, asterisked amino acids). In the absence of DNA, we observed a smear of peptides smaller than 10 kDa that were due to digestion of the unbound bZIP domain, which has a high number of trypsin sites (Fig. 4C, lane 3). The smear of low molecular weight bands was absent in the DNA-bound sample, consistent with the idea that this region is protected from proteolysis upon DNA binding (Fig. 4C, lanes 4–6). Sequence analysis of relevant peptides allowed assignment of the specific fragments within pCREB. Pertinent bands observed by Coomassie staining in Fig. 4C are numbered and the corresponding fragments are shown in Fig. 4D. Interestingly, we found that digestion of DNA-bound pCREB yielded two prominent fragments (bands 3 and 6) that were underrepresented in the free pCREB reactions. These fragments arose from cleavage at a site near the N-terminal ends of KID and bZIP, respectively (Fig. 4D).

There are three consecutive trypsin-sensitive basic residues beginning at Arg270, none of which make interactions with the CRE DNA (53). DNA binding enhanced digestion at this site, indicating a DNA-induced conformational change. The N-terminal fragments formed from digestion at the Arg270 region (band 6) are represented as bands 2, 4, and 5. A more significant difference between the pattern of digestion observed in free versus DNA-bound pCREB was enhancement in the intensity of band 3 (Fig. 4C, lanes 4, 5 versus 2, 3). Surprisingly, sequence analysis revealed that this peptide begins at Arg111, and the fragment size indicates that it extends from the KID region through the C terminus. This indicates that DNA binding by pCREB enhances trypsin cleavage at a site eight residues N-terminal to the site of PKA phosphorylation, consistent with a conformational change in pKID upon DNA binding. This conformational change may account for the DNA-dependent inhibition of pCREB dephosphorylation. KIX binding to pCREB in solution fully protected KID from trypsin cleavage (Fig. 4C, band 3, lanes 7–9). As expected, in the pCREB*KIX reaction we observed a smear of fragments that corresponded to digestion of the unbound bZIP domain (Fig. 4C, lanes 7–9). We also observed KIX protection of pCREB throughout KID and the entire N-terminal two-thirds of pCREB, as bands 1 and 2 each result from digestion within bZIP.

It is noteworthy that we observe both qualitative and quantitative changes in our trypsin digestion patterns which differ relative to those presented in previously published reports (18, 20, 22).

**Proteolysis Reveals a DNA-dependent Alteration in pCREB Conformation**—Previous studies have suggested that CREB is a natively unfolded protein in solution (18, 21). FoldIndex, one of many protein folding prediction programs, uses a Uversky algorithm based on the net charge and average residue hydrophobicity of a sequence to predict if a protein is natively unstructured (36, 37). Interestingly, this program predicts that CREB<sub>327</sub> is largely ordered, with alternating folded and unfolded regions (Fig. 4A). Only two distinct regions are predicted to be predominantly unfolded, mapping to KID and bZIP, consistent with reports that both the KID and bZIP regions have induced structure upon binding to KIX and DNA, respectively (13, 17, 18, 20, 22).

**FIGURE 3. CRE DNA and the KIX domain inhibit CREB dephosphorylation.**

A, pCREB dephosphorylation is inhibited by DNA in a sequence-specific manner. pCREB (25 nm) was preincubated with the indicated DNA (100 nm), GST, or GST-KIX<sub>aa88 – 683</sub>(0.8 μM each). Following the 20 min preincubation, PP1 was added for an additional 30 min. Proteins from the same samples were resolved on two gels, run in parallel, and analyzed by immunoblot with a CREB-specific antibody (to detect total CREB protein, CREB<sup>†</sup>) (bottom panel). B, CRE DNA and KIX protect CREB from PP1 dephosphorylation. [32P]CREB (25 nm) was preincubated for 20 min with a ds CRE oligonucleotide (CRE 1, 100 nm) and/or KIX (10 μM), followed by incubation with PP1 for the times indicated. The proteins were analyzed by SDS-PAGE and autoradiography. C, graphical representation of the data in Fig. 3B. The percentage of dephosphorylation of [32P]CREB in the presence of PP1, KIX, and DNA was calculated relative to the intensity of [32P]CREB in the absence of PP1, which was set to 0. The graph represents the results of three independent dephosphorylation assays. D, CRE DNA protects full-length CREB homodimers and CREB:bZIP heterodimers from PP1 dephosphorylation. Full-length [32P]CREB (FL) (25 nm) and [32P]CREB:bZIP (FL:bZIP) (12.5 nm each) were preincubated with excess ds CRE oligonucleotide (CRE 1, 100 nm) and/or KIX (10 μM), followed by incubation with PP1 for the times indicated. The reactions were analyzed by SDS-PAGE and autoradiography. To ensure a comparable signal, an equal amount of total labeled CREB was loaded in each lane on the gel. The decreased level of CREB dephosphorylation observed in this figure, relative to Fig. 3B, is caused by unavoidable variation in the activity of the PP1 used in each experiment.
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FIGURE 4. Proteolytic cleavage reveals a DNA-dependent alteration in pCREB conformation. A, FoldIndex of CREB327. Folding predictions were plotted with the default window size of 51 amino acids and calculated by the Uversky method using average residue hydrophobicity and net charge. Values above zero (green) represent regions predicted to exhibit ordered structure. Values below zero (red) represent regions predicted to be unfolded. B, sequence of CREB327. Amino acids in red and green correspond to unfolded and ordered regions, respectively, as displayed by FoldIndex. The KID region and bZIP domain are underlined. Asterisks represent potential tryptic digestion sites, with prominent tryptic cleavage sites indicated in red. The position of the 14 amino acid α domain, which distinguishes CREB327 from CREB341, is indicated. Boxed amino acids indicate the sequence deleted in CREB341Δ121–125 (see Fig. 7). C, tryptic proteolysis. Reactions were carried out in the presence of trypsin (1:100 w/w, trypsin:CREB) for the times indicated. The proteins were resolved by 10% SD-Tricine SDS-PAGE and visualized by Coomassie Brilliant Blue staining. Prominent bands are numbered.

C.

| Time | 0 | 1 | 5 | 10 | 30 |
|------|---|---|---|----|----|
| 1   | N |  |   |    |    |
| 2   | N |  |   |    |    |
| 3   | N |   |   |    |    |
| 4   | N |   |   |    |    |
| 5   | N |   |   |    |    |
| 6   | N |   |   |    |    |
| 7   | N |   |   |    |    |

D.

| full-length: | N |  |   |  |    |   |
|-------------|---|---|----|---|----|---|
| 1           | N |   |   |    |    |    |
| 2           | N |   |   |    |    |    |
| 3           | N |   |   |    |    |    |
| 4           | N |   |   |    |    |    |
| 5           | N |   |   |    |    |    |
| 6           | N |   |   |    |    |    |
| 7           | N |   |   |    |    |    |

PKA Phosphorylation Is Not Inhibited by DNA Binding—The KIX domain of CBP/p300 interacts with DNA-bound pCREB both in vitro and in vivo, and this interaction is critical to transcriptional activation (13, 54, 55). While DNA-bound pCREB is resistant to dephosphorylation, we still observe KIX binding, indicating that pKID is fully accessible to the KIX domain of CBP/p300 (data not shown). To further explore the conformation of KID in DNA-bound CREB, we performed kinase assays to determine whether Ser119 is accessible for phosphorylation by PKA. CREB was preincubated in the absence or presence of the fragment carrying four reiterated copies of a CRE cloned upstream of a minimal promoter. Unbound CREB was removed from the immobilized template, and both reactions were incubated with PKA and [γ-32P]ATP. Proteins were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue staining (Fig. 5A, lower panel) and autoradiography (Fig. 5A, upper panel). We found CREB was phosphorylated by PKA in both the absence and presence of DNA.

The relevance of these data to the physiological function of CREB prompted us to test whether CREB was similarly phosphorylated by PKA when bound to chromatin templates. For direct comparison, purified Drosophila core histones were assembled onto the same immobilized promoter fragment using the recombinant assembly proteins Acf1/ISWI and Drosophila NAP-1 (39). These assembly proteins are sufficient for the ATP-dependent formation of evenly spaced nucleosomal arrays (56). Micrococcal nuclease assays were performed to verify
migrates with reduced mobility relative to CREB/DNA (Fig. 5).

However, electrophoretic mobility shift of a protein, which would be predicted to result in increased CREB. Phosphorylation increases the net negative charge of DNA-bound unphosphorylated and phosphorylated CREB. The gel was processed as described in Methods.

This raised the question as to whether phosphorylation per se may also be responsible for inducing a conformational change within KID. We investigated potential differences in the conformation of DNA-bound unphosphorylated and phosphorylated CREB. Phosphorylation increases the net negative charge of a protein, which would be predicted to result in increased mobility in a native gel. However, electrophoretic mobility shift assays (EMSA) demonstrated that the pCREB/DNA complex migrates with reduced mobility relative to CREB/DNA (Fig. 6A), consistent with previous studies (22, 57). This data suggests that phosphorylation induces a structural change that increases the apparent size or shape of the CREB/DNA complex. To determine whether this reduced mobility resulted from shape-altering intermolecular interactions between monomers in the DNA-bound CREB homodimer complex, we again formed heterodimeric complexes with an equal molar ratio of full-length CREB (or pCREB) and the bZIP domain, bound to the probe. Results from the EMSA show that, like the full-length homodimer, phosphorylation also reduces the mobility of the pCREB/bZIP heterodimer (Fig. 6B, lanes 4 and 5). Therefore, the apparent increase in the size of pCREB/DNA complex does not result from intermolecular contacts between the phosphorylated dimers, but rather originates within a single CREB monomer by phosphorylation of KID.

To further characterize the molecular size and/or shape of the complexes, CREB and pCREB were bound to a 21-bp CRE oligonucleotide and compared by size-exclusion chromatography using an analytical Superdex 200 10/300 GL column. A small but highly reproducible difference in the elution profiles of CREB/DNA and pCREB/DNA (VE = 9.87 ml and VE = 9.74 ml, respectively, Fig. 6C) is consistent with the larger apparent size of pCREB/DNA observed by EMSA. Interestingly, the CREB/pCREB/DNA heterodimeric complex exhibited an intermediate elution profile (VE = 9.80 ml). The observation of a distinct, intermediate peak produced by the heterodimer suggests that the three dimeric forms of CREB present in the sample (CREB:CREB, pCREB:pCREB, CREB: pCREB) exist in a rapid equilibrium. Based on a calibration curve using globular molecular weight standards, both the CREB/DNA and pCREB/DNA complexes eluted from the column at unusually high apparent molecular weights (approximately 480 and 500 kDa, respectively). The calculated molecular weight of the CREB dimer bound to the CRE DNA is ~84 kDa. We have extensively analyzed the stoichiometry and solubility of CREB/DNA complexes by EMSA, gel filtration, and analytical ultracentrifugation, and have confirmed that CREB is bound to DNA as a dimer and is free of aggregation normally attributed to oxidation and disulfide bond formation (data not shown). Therefore, the large apparent size of both complexes likely reflects an extraordinarily asymmetric, oblong structure of both CREB and pCREB bound to DNA. CREB, in the absence of DNA, elutes at ~300 kDa (data not shown).

To further assess the phosphorylation-induced conformational change in the CREB/DNA complex, we compared the trypsin cleavage pattern of CREB and pCREB bound to the consensus CRE. In solution, CREB phosphorylation produced a slight but significant resistance to proteolytic cleavage relative to unphosphorylated CREB, consistent with previously published studies (18, 22, 23) (data not shown). We observed an increase in the accumulation of a trypsin-resistant fragment in the pCREB/DNA reactions relative to the CREB/DNA reactions (Fig. 6D, band 2). Notably, this fragment arose from decreased sensitivity to trypsin at the site N-terminal to Ser119 (Arg111) and increased digestion at the site immediately N-terminal to the bZIP domain of CREB (~Arg279) (Fig. 6E). These data are consistent with a phosphorylation-induced conformational change that can be detected near the C-terminus of CREB. This conformational change is congruent with the increase in size and shape observed in the EMSA and size-exclusion chromatography.
DNA Binding Inhibits CREB Dephosphorylation

Basic Amino Acids in KID Participate in Forming a More Compact Structure—Finally, we investigated a possible molecular mechanism for the altered conformation of the phosphorylated form of the CREB/DNA complex. A cluster of seven basic amino acids flank the PKA phosphorylation site in KID (Fig. 4B). In the absence of the negatively charged phosphate group, these basic amino acids may interact with neighboring acidic residues, or with the DNA, leading to the more compact structure observed with unphosphorylated DNA-bound CREB. Phosphorylation at Ser119 may disrupt these putative intramolecular charge interactions, thus promoting a conformation that enhances the apparent size or shape of the molecule. If our hypothesis is correct, deletion of nearby basic amino acids in KID should mimic the conformation adopted by phosphorylation, resulting in an apparent increase in the molecular size of the complex.

To test this hypothesis, we purified a mutant CREB341 Δ121–125 with a deletion of three consecutive basic amino acids (KRR) which are farthest from Ser133 and do not interfere with the PKA recognition site (Fig. 4B) (23, 58). In our previous experiments we used CREB327, however, this mutant was prepared in CREB341. Despite the small differences in molecular weight of the three CREB proteins, all migrated comparably on SDS-PAGE (Fig. 7A). However, the CREB341 Δ121–125/DNA complex migrated with reduced mobility in an EMSA relative to the complexes formed with CREB327 and CREB341 (Fig. 7B, lanes 1–3). As predicted, CREB341 Δ121–125/DNA migrated on the native polyacrylamide gel similar to pCREB/DNA, suggesting that deletion of these basic amino acids analogously enhances the apparent size or shape of the molecule (Fig. 7B, lanes 5 and 6). Phosphorylation of CREB341 Δ121–125 further reduced its mobility in an EMSA (Fig. 7B, lanes 6 and 7), which may be attributed to basic amino acids that were not deleted. Analytical size-exclusion chromatography of the CREB341 Δ121–125/DNA complex produced an elution profile nearly identical to that observed for pCREB/DNA (compare Figs. 7C and 6C). The difference in elution profiles observed between unphosphorylated and phosphorylated CREB327/DNA was also observed between CREB341/DNA and CREB341 Δ121–125/DNA (Ve = 9.83 ml and Ve = 9.73 ml, respectively). Furthermore, the heterodimeric complex also exhibited an intermediate elution profile (Ve = 9.79 ml). Because the Δ121–125 mutation is expressed in the CREB341 isoform, we also compared the chromatographic profiles of the unphosphorylated, phosphorylated, and heterodimeric forms of CREB341/DNA. Fig. 7D shows that the elution profile of pCREB341/DNA is identical to that of CREB341 Δ121–125/DNA (Ve = 9.73). Together, these data indicate that deletion of the basic amino acids mimics the conformational change in CREB that is induced upon phosphorylation. The Δ121–125 mutation in CREB341 did not affect phosphorylation by PKA or resistance to dephosphorylation by nuclear extract (data not shown).
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These data are consistent with a model in which basic amino acids in KID participate in electrostatic interactions that confer a more compact structure in CREB. Upon phosphorylation, these interactions are disrupted, and pKID adopts a more open conformation that facilitates the binding of the KIX domain of CBP/p300.

DISCUSSION

In this study, we were interested in biochemically characterizing the properties of the phosphorylated, DNA-bound, transcriptionally active form of CREB. We found that protein phosphatase PP1 dephosphorylates pCREB when free in solution; however, sequence-specific DNA binding inhibits pCREB dephosphorylation. DNA-dependent resistance to dephosphorylation appears to be conferred by a conformational change that renders pKID inaccessible or unrecognizable to PP1, while KID remains fully accessible to PKA. Most interestingly, we find that in addition to DNA binding, phosphorylation induces a further conformational change in DNA-bound CREB. This was demonstrated by an increase in the size or asymmetry of the phosphorylated complex and by a qualitative change in the proteolytic digestion pattern. Deletion of basic amino acids in KID mimic the phosphorylation-induced conformational alteration, suggesting that intramolecular interactions promote a more compact structure in CREB that is disrupted upon phosphorylation. Together, our data support the existence of three distinct CREB structures: (i) pCREB free in solution, (ii) CREB bound to DNA, and (iii) pCREB bound to DNA. These data are unexpected, as previously published studies have suggested that the only significant structural alterations in the otherwise unstructured protein are induced solely within the bZIP domain upon DNA binding and within the KID region upon KIX binding (13, 17, 18, 20, 22).

We observe a global structural change in pCREB upon binding to CRE DNA. This conformational alteration is evident from the proteolytic cleavage pattern showing DNA-dependent hypersensitivity at a trypsin site located within the KID region. The fragment produced from this cleavage encompasses not only the DNA-bound bZIP domain but the full KID region as well. In further support of a structural connection between bZIP and KID, the binding affinity of full-length pCREB for KIX has been shown to be approximately 200-fold higher than that of pKID alone (13). This indicates that the authentic high affinity pKID structure may be influenced by structural cues that originate within the DNA-bound bZIP domain. Moreover, indications of structural changes throughout CREB have been previously published, most notably in a study suggesting that the lack of the bZIP domain influences the secondary structure of the activation domain (18). Similarly, studies on various CREB domains have implicated a role for the Q2 domain and sequences N-terminal to the KID region affecting the DNA binding affinity of CREB (29, 52). Therefore, the KID region may not function as an independent domain, but rather cooperatively with bZIP via a conformational change that originates within bZIP upon DNA binding. This signal may be transmitted through the Q2 domain, and thus influence the structural properties of this activation domain of CREB. Interestingly, Q2 alone has significant secondary structure, as predicted by the FoldIndex plot of CREB (Fig. 4A). This is consonant with our data, as secondary structure within Q2 may enable propagation of the signal from bZIP into KID.

It is seemingly confounding that DNA binding confers a structure in KID that enhances the accessibility to PKA while...
simultaneously rendering pKID inaccessible to PP1. In essence, the structure of the DNA-bound KID region recognized by these two enzymes may be affected by its phosphorylation status; KID and pKID are in two different DNA-dependent conformations. In further support of a phosphorylation-induced structural change in DNA-bound CREB, we observe enhanced proteolytic cleavage at the Q2 and bZIP junction, suggesting that phosphorylation in KID alters the conformation of Q2 and bZIP. Although both CREB and pCREB bound to DNA elute from the gel filtration column at an unusually large apparent molecular weight, perhaps reflecting an extraordinarily asymmetric or oblong structure, a further increase in apparent size was observed with the phosphorylated complex. In support of these observations, visualization of CREB dimers by electron microscopy also suggests an increase in the area between the two monomers upon phosphorylation (57). Similarly, Ferguson plot analysis has shown an increase in the spherical size and net positive surface charge of the CREB/DNA complex upon phosphorylation (29). Together, this indicates that phosphorylation promotes a structural change in the CREB/DNA complex that leads to a less compact or more asymmetric tertiary structure.

The structural changes induced by both DNA binding and phosphorylation originate from within a single CREB molecule and are not caused by intermolecular interactions between the DNA-bound dimer. We found that DNA-bound pCREB/bZIP heterodimers exhibit equivalent protection from PP1 dephosphorylation as full-length homodimers and both display a comparable, less compact tertiary structure than that of unphosphorylated CREB complexes. The notion that the intrinsic structure of one monomer in the DNA-bound pCREB dimer is independent of intermolecular interactions with its partner is supported by structural studies showing a single phosphorylated KID interacts with a single KIX (24, 57). Furthermore, deletion of basic amino acids near Ser133 in CREB341Δ121–125, which resulted in a less compact tertiary structure than that exhibited by pCREB, suggests that the negative charges from the phosphate group disrupt intramolecular electrostatic interactions between these basic residues and neighboring acidic residues. Indeed, intramolecular interactions between the basic residues adjacent to Ser133 of CREB341 and the acidic residues from amino acids 140–144 (DLSSD) have been shown to stabilize the secondary structure of KID (23). An additional highly acidic stretch of amino acids from 138–145 in CREB327 (EEKSEELEE) may also make similar contacts that render a more compact structure in unphosphorylated CREB.

Phosphorylation and subsequent dephosphorylation serves as a cellular mechanism for the regulation of many signal dependent genes. CREB is a prototypic example of such a phosphorylation-dependent transcription factor. During the burst phase, CREB phosphorylation stimulates transcription of target genes which is followed by an attenuation phase characterized by pCREB dephosphorylation (10, 11). Because our data indicate that DNA binding reduces the rate of pCREB dephosphorylation relative to pCREB in solution, the attenuation phase may simply be defined by this change in dephosphorylation kinetics. In other words, because pCREB is constitutively bound to DNA in vivo (2–4), it possesses a phosphatase-resistant structure which may prolong the transcriptionally active state. Immediately following signal-induced CREB phosphorylation, the DNA-bound pCREB rapidly recruits the coactivators CBP/p300, TORCs, and additional factors essential for transcriptional progression (2). The global change in tertiary structure induced by phosphorylation of the CREB/DNA complex may enhance recruitment of CBP/p300 in addition to the recruitment of other transcription factors. Most notably, the cooperativity between phosphorylated KID (which recruits CBP/p300) and the Q2 domain (which recruits TFIIID) may be potentiated by the conformation changes induced by DNA binding and phosphorylation. In conclusion, these biochemical studies provide a framework for enhanced understanding of CREB transcription function in vivo.

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