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Structural basis for graded inhibition of CREB:DNA interactions by multi-site phosphorylation

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Abstract

Phosphorylation of the kinase-inducible domain (KID) of the cyclic AMP response element binding transcription factor (CREB) regulates its function through several mechanisms. Transcriptional activation occurs following phosphorylation at serine 133, but multisite phosphorylation in a neighboring region termed the CK cassette, residues 108–117, results in inhibition of CREB-mediated transcription. A molecular-level understanding of the mechanism of these opposing reactions has been lacking, in part because of the difficulty of preparing multiply-phosphorylated CREB *in vitro*. By substitution of a single residue, we have generated an engineered mammalian CREB in which the CK cassette can be phosphorylated *in vitro* by casein kinases and have characterized its interactions with cyclic AMP response element (CRE) DNA. Phosphorylation of the CK cassette promotes an intramolecular interaction between the KID domain and the site of DNA binding, the basic region of the C-terminal basic leucine zipper (bZip) domain. Competition between the phosphorylated KID domain and DNA for bZip binding results in lowered affinity of CREB for DNA. The binding free energy calculated from the dissociation constant is directly proportional to the number of phosphate groups in the CK cassette, indicating that the DNA binding is regulated by a rheostat-like mechanism. The rheostat is modulated by variation in the concentration of cations such as Mg^{2+} and by alternative isoforms such as the natural CREB isoform that lacks residues 162–272. Multisite phosphorylation of CREB represents a versatile mechanism by which transcription can be tuned to meet the variable needs of the cell.

Graphic Abstract

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Notes

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ASSOCIATED CONTENT

Supporting Information. Figures S1–S7 showing labeled HSQC spectra of KID, bZip, and full-length CREB, mutation patterns, bar graphs showing effects on DNA affinity of increasing phosphorylation of CREB, and changes in fluorescence anisotropy of DNA upon addition of CREB constructs, together with a table showing all dissociation constants. This supplementary material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes. Chemical shifts for the non-phosphorylated and CKII-phosphorylated V118D KID domain have been deposited in the BioMagResBank under accession codes 27648 and 27647, respectively.

Keywords

transcriptional activation; intrinsically disordered protein; competitive binding; protein-protein interactions; NMR

Introduction

The cyclic AMP-response element (CRE)-binding protein (CREB; UniprotKB P15337) is a ubiquitously expressed transcription factor implicated in many cellular processes including cell survival and proliferation, hematopoiesis, long-term memory, immune response, and glucose homeostasis.^{1–4} Dysregulation of CREB pathways is associated with neuropathological disorders and oncogenesis.^{5–7} CREB is composed of 4 domains: two glutamine rich domains (Q1 and Q2), a kinase inducible transcriptional activation domain (KID), and a basic region leucine zipper (bZip) DNA binding and dimerization domain (Fig. 1). The Q1, KID, and Q2 domains are predicted to be intrinsically disordered.⁸ Dimerization of CREB through the bZip domain yields homodimers that bind to numerous target genes containing CRE sequences in their promotor regions. CREB activity is regulated in response to cellular and extracellular stimuli, including elevated levels of cyclic-AMP (cAMP). Canonical activation of CREB proceeds via protein kinase A (PKA)-dependent phosphorylation at Ser133, which facilitates recruitment of the CBP/p300 transcriptional coactivators and leads to enhanced transcription.^{9,10} In contrast to the activation by phosphorylation of Ser133, CREB transcriptional activity can be inhibited in response to genotoxic stress via processive multisite phosphorylation of a conserved cluster of residues, S108, S111, S114, S117, and S121, by the casein kinases (CKI and CKII) and the ataxia-telangiectasia-mutated (ATM) kinase.^{11,12} These residues are collectively referred to as the CK/ATM cluster (Fig. 1). S108, S111, S114, S117 (hereafter called the CK cassette) are also phosphorylated during cell growth, independently of ATM.¹³ Multisite phosphorylation of the CK/ATM cluster inhibits CREB-mediated transcription, coactivator and DNA binding, and chromatin occupancy.^{11,12} DNA binding is inhibited in a graded manner, dependent on the number of sites phosphorylated in the CK/ATM cluster.¹¹

In the present work, we have used NMR and fluorescence anisotropy to probe the mechanism by which phosphorylation of the CK cassette modulates the DNA binding activity of CREB. Although in-cell studies have revealed the functional outcome of multisite phosphorylation of CREB, quantitative biophysical and thermodynamic data have not been reported, in part due to the difficulty of phosphorylating the CK cassette or the CK/ATM cluster *in vitro*.¹¹ To overcome this problem, we engineered a CREB construct based on the sequence of a *Drosophila* homolog¹⁴ that can be phosphorylated in the CK cassette *in vitro*

using a single kinase. This construct facilitated biophysical studies of the effects of multisite phosphorylation on CREB binding to DNA and allowed us to determine the contributions of individual phosphorylation events to the overall response. We show that phosphorylation of the CK cassette directly reduces the DNA-binding affinity of CREB. Each successive phosphorylation event further reduces DNA binding affinity, consistent with a rheostat model. Further, we show that phosphorylation of the CK cassette results in interactions of this region with the basic leucine-zipper DNA-binding domain of CREB, suggesting a structural basis for the phosphorylation-dependent reduction of DNA binding affinity.

Materials and Methods

Protein Expression and Purification

Full-length CREB from *Rattus norvegicus* (residues 1–341) with mutations (via QuikChange) of V118 and the 4 cysteine residues (V118D, C90A, C300S, C310S, C337S; termed CREB^{D,4}) was cloned into a pET21a vector with a tobacco etch virus (TEV) protease cleavable N-terminal His₆-GB1 tag. The Cys to Ser mutations in the bZip domain increase the solubility without affecting DNA binding.¹⁵ CREB was expressed in *Escherichia coli* BL21(DE3) DNAY cells grown at 37°C in LB media or minimal M9 media supplemented with ¹⁵N-ammonium acetate and, when used for backbone resonance assignment, ¹³C-D-glucose. Cells were grown to OD₆₀₀ 0.6 before induction with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and overnight incubation at 25°C. Cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 1 M NaCl, supplemented with Pierce EDTA-free protease inhibitor) and lysed by sonication. CREB was purified using His-cOmplete resin (Roche) and eluted with lysis buffer supplemented with 0.2 M imidazole. TEV protease was added to the eluted protein at a 1:100 mole ratio. Cleaved protein (with an N-terminal Gly-Ser-His tag) was dialyzed overnight against 4L of buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2mM DTT, then diluted 3-fold with 20 mM Tris-HCl (pH 7.5) and further purified by ion exchange on a Hi-Trap SP column (GE Healthcare). The purity of the eluted protein was confirmed by SDS-PAGE and mass spectrometry. The KID domain construct (residues 85–160 of rat CREB, with C90A and V118D mutations) was prepared similarly to full-length CREB, but was purified using reverse-phase HPLC instead of the Hi-Trap SP column. Homogeneity was confirmed by SDS-PAGE and mass spectrometry. The bZip domain of CREB (residues 270–341 of rat CREB, with C300, C310, and C337 mutated to serine) was prepared similarly to full-length CREB but with buffers containing 20 mM MES (pH 6.0) instead of Tris-HCl. A phosphorylation-mimic variant of full-length V118D CREB was prepared by introducing glutamate mutations at all of the phospho-acceptor sites in the CK cassette (S108E, S111E, S114E, and S117E plus T119E since we observed that T119 is also phosphorylated by CKII). A series of CREB mutants with a decreasing number of CKII phosphorylation sites was generated by introducing Ser/Ala mutations in the CK cassette (CREB^{D,3}, S108A; CREB^{D,2}, S108A/S111A; CREB^{D,1}, S108A/S111A/S114A; CREB^{D,0}, S108A/S111A/S114A/S117A) within the V118D, C90A, C300S, C310S, C337S CREB variant. The alanine and glutamic acid variant proteins were purified as described above.

Protein Phosphorylation

Phosphorylation reactions for NMR assignments and titrations were carried out in phosphorylation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) supplemented with 2 mM DTT, 9 mM ATP, 10 mM MgCl₂, and 2000 units of CKII (New England Biolabs) at 30°C. Phosphorylation was confirmed by recording ¹H-¹⁵N HSQC spectra for both full-length V118D CREB and the isolated V118D KID domain construct, and also by mass spectrometry for the KID domain. Prior to NMR experiments, samples were exchanged into NMR buffer (see below) using a NAP column. Small scale phosphorylation reactions for affinity measurements and the spectra shown in figures 5 and S3 were carried out using 40 μM protein in phosphorylation buffer supplemented with 2 mM DTT, 10 mM MgCl₂, 3 mM ATP, and 500 units of CKII. The reaction mixtures were incubated overnight at 30°C. Non-phosphorylated protein controls were obtained by incubating samples in phosphorylation reaction mixture from which CKII was omitted.

NMR Spectroscopy

For backbone resonance assignments, ¹³C,¹⁵N-labeled C90A/V118D KID and phosphorylated C90A/V118D KID (henceforth termed p^{CK}KID) peptides were prepared in 20 mM MES (pH 6.0), 100 mM NaCl, 10% D₂O. Assignments of the ¹³C,¹⁵N-labeled bZip domain were made in 20mM MES buffer, pH 6.0, containing 50mM NaCl. Samples for NMR titrations and samples of full-length V118D CREB^{D,4}, CREB^{D,2}, and CREB^{D,0} with and without CRE DNA were prepared in 20 mM MES (pH 6.0), 50 mM NaCl, 1.5 mM NaN₃, 5% D₂O. NMR spectra were recorded at 25°C on Bruker Avance 600 and 900 MHz spectrometers. Spectra were processed with NMRPipe¹⁶ and analyzed using Sparky,¹⁷ within the NMRBox resource.¹⁸ Backbone resonances of C90A/V118D KID and CKII-phosphorylated C90A/V118D KID (p^{CK}KID) were assigned using 3D HN(CA)CO, HNCO, CBCA(CO)NH, HNCACB, and 2D ¹H-¹⁵N HSQC experiments.^{19,20} The pSer and pThr cross peaks of p^{CK}KID appear at similar chemical shifts in the spectrum of DNA-bound, CKII-phosphorylated, full-length V118D CREB^{D,4} at the same pH, allowing assignments to be transferred to the full-length protein. The assignments were confirmed by Ser/Ala mutagenesis (Fig. S3). Backbone assignments for p^{CK}KID in complexes with the bZip domain were transferred from those of the isolated p^{CK}KID using ¹H-¹⁵N HSQC titrations. Assignment of the Asn293 side chain N^δH cross peaks in the bZip was accomplished by N293S mutagenesis. Titrations of KID and bZip were monitored by two-dimensional ¹H-¹⁵N HSQC spectra. Chemical shift deviations (δ_{av}) between the free and bound KID were calculated using:

$$\Delta\delta_{av} = \sqrt{(\Delta\delta_{HN})^2 + (\Delta\delta_N/5)^2}$$

where δ_{HN} and δ_N are the difference between free and bound amide proton and nitrogen chemical shifts.²¹ The high-affinity CRE DNA used for NMR experiments was ordered from IDT (5' - CCTTGGCT**GACGTC**AGCCAAG-3' and its complementary sequence; the somatostatin CRE site is shown in bold). The DNA duplex was annealed as described below.

Fluorescence Anisotropy Binding Assays

Fluorescence anisotropy measurements were performed on a PC1 photon-counting steady-state fluorimeter (ISS) and a Fluorolog-3 spectrofluorometer (Horiba). Fluorescently labeled oligonucleotide and its unlabeled complementary sequence containing a half-site CRE (5'-Cy5-ATCTGCGTCAGAGT-3' and 5'-ACTCTGACGCAGAT-3'; the CRE site is in bold) were purchased from IDT. DNA was resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl. The complementary sequences were mixed in 1:1 mole ratio and annealed by heating to 94°C for 2 minutes in a water bath followed by gradual cooling. CREB constructs in phosphorylation buffer were titrated into fluorescently labeled DNA duplex in Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, with either 0 mM or 5 mM MgCl₂ (see text) at 25°C. Titrations with the phosphorylation-mimic glutamate mutant, with the isolated bZip domain, and with the bZip domain plus V118D KID or p^{CK}KID, were performed with buffer containing 200mM NaCl. Samples were mixed and incubated for 10 minutes prior to measurement. Binding data were fitted to the standard equation for a one-site binding model:

$$Y = Y_{free} + \frac{(Y_{bound} - Y_{free})((K_d + P_0 + L_0) - ((K_d + P_0 + L_0)^2 - 4P_0L_0)^{0.5})}{2L_0}$$

where Y is the measured anisotropy, Y_{free} and Y_{bound} are the anisotropy of free and bound ligand, L_0 = ligand concentration, P_0 = protein concentration, and K_d is the dissociation constant. Graphs were plotted using GraphPad Prism 7 (GraphPad Software, La Jolla California USA).

Results

Design of constructs for efficient phosphorylation of CREB *in vitro*

Our initial attempts to phosphorylate the CK cassette *in vitro* using CKI and CKII kinases resulted in only partial phosphorylation of a subset of residues. This is consistent with prior observations that CKII was only able to phosphorylate two sites on CREB.²² However, a *Drosophila* homolog of mammalian CREB, dCREB2, can be phosphorylated *in vitro* at residues homologous to the mammalian CK cassette using CKI or CKII alone.¹⁴

Comparison of the primary sequence of mammalian CREB with dCREB2 shows differences following S117, the last residue of the CK cassette, which could affect its phosphorylation efficiency (Fig. 1). The last serine in the dCREB2 CK cassette is followed by an acidic patch (Asp-Asp-Asp), which creates a highly efficient CKII recognition site. By contrast, in vertebrate CREB, the corresponding residues (Val-Thr-Asp) form a less effective CKII recognition site, likely explaining the decreased phosphorylation efficiency. Engineering of a mammalian (rat) CREB construct (hereafter CREB^{D,4}) containing a phosphorylation-enhancing mutation, V118D, based on the sequence of dCREB2 (Fig. 1) renders S117 an optimal CKII site. NMR analysis (Fig. 2A) showed that the CREB^{D,4} construct could be fully phosphorylated at the 4 residues of the CK cassette with CKII alone, both in an isolated KID domain construct (residues 85–160) (Fig. 2A and Fig. S1) and in full-length CREB (Fig. 2B). Resonance assignments for the isolated V118D KID domain, before and after

treatment with CKII, were made using triple resonance experiments with a uniformly ^{13}C , ^{15}N labeled sample and transferred to the full-length CREB^{D,4}, as described in *Materials and Methods*. The lack of dispersion in the ^1H dimension of the ^1H - ^{15}N HSQC spectrum confirms that the V118D KID domain, like wild type KID,²³ is intrinsically disordered in both its CKII-phosphorylated and unphosphorylated states. Interestingly, several residues of the KID domain outside the CK cassette, Thr119, Ser143, and Ser156, are also fully or partially phosphorylated. These residues have been reported to be CKII sites,²⁴ but are often not discussed in reports that use antibodies specific for the phosphorylated CK cassette, which would be unable to detect additional phosphorylation sites. Introduction of the V118D mutation enables full phosphorylation of the CK cassette in mammalian CREB *in vitro* for biophysical studies of the multisite phosphorylation-dependent regulatory pathway.

To assess the role of multisite phosphorylation, additional CREB constructs with decreasing numbers of phosphorylation sites were designed: CREB^{D,3} (S108A), CREB^{D,2} (S108A, S111A), CREB^{D,1} (S108A, S111A, S114A), and CREB^{D,0} (S108A, S111A, S114A, S117A), where the superscript indicates the number of residues available for phosphorylation in the CK cassette (Fig. S2). The substitution of these serines in the CK-cassette was confirmed by NMR spectroscopy (Fig. S3).

Interaction of the phosphorylated CK cassette with the bZip domain.

The major effect of multisite phosphorylation of the CK cassette is a large increase in local negative charge. We hypothesized that the negatively charged, phosphorylated CK cassette might interact with the positively charged basic region of the bZip DNA binding domain. To probe these interactions, we performed a series of NMR experiments where ^{15}N -labeled V118D KID domain peptide (residues 85–160 of human CREB), containing either a phosphorylated or non-phosphorylated CK cassette, was titrated with unlabeled bZip. Titration of bZip into ^{15}N -labeled p^{CK}KID causes shifts, in fast exchange on the chemical shift time scale, of many amide cross-peaks in the ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectrum, indicating that the two domains bind to form a complex (Fig. 3A, S4A). In contrast, upon addition of bZip to non-phosphorylated V118D KID, there are minimal changes in the HSQC spectrum, suggesting that KID interacts only weakly with the bZip domain in the absence of phosphorylation (Fig. 3B, S4B). A plot of the weighted average ^1H and ^{15}N chemical shift changes (δ_{av}) observed upon titration with bZip (Fig. 3C and D) shows that phosphorylated KID interacts with bZip mainly through the CK cassette and the surrounding residues. In the absence of phosphorylation, V118D KID shows only weak interactions with bZip through its C-terminal region, which is rich in glutamic acid residues ($^{152}\text{EEEEKSEEET}^{160}$).

The reverse titration, in which unlabeled V118D KID or p^{CK}KID was added to ^{15}N -labeled bZip, (Fig. 4; full HSQC spectra are shown in Fig. S5) showed that the basic region of the bZip domain is the primary site of interaction. Under the experimental conditions used, pH 6.0 and 50 mM NaCl, the amide cross peaks of most residues in the leucine zipper are broad and weak and were not assigned. Although poorly dispersed, many backbone resonances associated with the disordered basic region, which mediates both specific and non-specific DNA interactions,²⁵ were assigned from triple resonance spectra. Upon addition of p^{CK}KID,

backbone amide cross peaks from residues 285–290 in the basic region shift in fast exchange and/or broaden (Fig. 4A, 4C). Shifts were also observed for arginine side chain resonances (labeled “Rsc” in Fig. 4B, Fig. S5C) and one of the N^δH cross peaks of the Asn293 side chain (labeled “N293sc”), which makes base-specific contacts in the CREB:CRE X-ray structure.²⁵ The shift in the bZip HSQC cross peaks is largely complete by 0.5:1 p^{CK}KID:bZip stoichiometry, with little further change as more p^{CK}KID is added (Fig. S5C). In contrast, when non-phosphorylated KID is titrated into bZip only minimal shifts are observed in amide or side chain cross-peaks (Figs. 4D–F), consistent with the weak interactions noted above between the acidic KID C-terminal region and the bZip. Likewise, only very small chemical shift changes ($\delta_{av} < 0.02$ ppm) were observed for the few assigned resonances of the leucine zipper (Ala316, Thr324, Asp341) upon addition of V118D KID or p^{CK}KID. Taken together, our NMR experiments show that, following multisite phosphorylation of the CK cassette, the p^{CK}KID peptide interacts preferentially with the basic region of the bZip dimer, in a 1:1 p^{CK}KID:bZip dimer stoichiometry. In other words, the basic regions of the two polypeptide chains of the bZip dimer synergize to bind a single molecule of p^{CK}KID.

Changes are also observed in the ¹H-¹⁵N HSQC spectrum of CKII-phosphorylated full-length CREB^{D,4} upon addition of high affinity palindromic somatostatin CRE DNA (Fig. 5). In a spectrum recorded in the absence of DNA, weak and broadened cross peaks are observed for pS108, pS111, pS114, pS117, and pT119, at the same chemical shifts as in the HSQC spectrum of the isolated p^{CK}KID peptide (Fig. 5A). Broadening of these resonances is attributed to intramolecular exchange, on a slow-intermediate time scale, between free and bZip bound states; given the stoichiometry determined using the isolated peptides, it is likely that at any instant only one of the two KID regions is bound to the bZip dimer, giving rise to broad and overlapped cross peaks that could not be assigned, while the other KID domain is free. Upon addition of cognate DNA, the pSer and pThr cross peaks sharpen and become more uniform in intensity, as in spectra of the isolated phosphorylated p^{CK}KID domain (Fig. 5A, B). These observations are consistent with a model in which multisite phosphorylation of the KID domain promotes intramolecular interactions that compete with DNA for binding to the basic region of the CREB dimer.

Effect of multisite phosphorylation on DNA binding.

DNA binding affinity was measured for full-length CREB constructs with 0, 1, 2, 3 or 4 phosphorylation sites in the CK cassette, using a fluorescence anisotropy assay where either phosphorylated or non-phosphorylated CREB was titrated into fluorescently-labeled DNA containing a CRE half-site (Fig. 6A). Experiments were performed in pH 7.5 Tris buffer containing 150mM NaCl. Without phosphorylation, these constructs have no significant difference in DNA binding affinity (average affinity for all non-phosphorylated constructs is 4 ± 2 nM, see Table S1). In contrast, the phosphorylated constructs show a steady decrease in DNA binding affinity with each additional phosphorylation event (Fig. 6B and Table S1), with each phosphorylation event decreasing the binding free energy by 0.5 kcal/mol (Fig. 6B inset). The energetic contributions of these phosphorylation events are similar to values previously reported for other systems where phosphorylation acts as a regulatory rheostat.^{26,27} The DNA binding affinity is reduced as much as 30-fold upon phosphorylation of all 4

CK cassette residues (Table S1). It should be noted that CKII phosphorylation of CREB^{D,0}, in which all of the serines in the CK cassette are mutated to alanine, decreases the DNA binding affinity by approximately 4–6-fold relative to the unphosphorylated protein. This likely arises from phosphorylation of additional sites outside the CK cassette (Fig. S3).

Magnesium modulates the dynamic range of binding affinity

The presence of magnesium can modulate the DNA binding affinity of CREB; depending on the specific DNA sequence used, a magnesium concentration in the range of 3 to 10 mM can either reduce, enhance, or have no effect on DNA binding.^{28,29} To determine the effect of magnesium on the phosphorylation-dependent inhibition of DNA binding, affinity measurements were repeated in the presence of 5mM magnesium. Similar to a previous report,²⁸ the increased concentration of magnesium reduced the binding affinity of non-phosphorylated CREB^{D,4} by almost 3-fold (Table S1). The increased magnesium concentration had the opposite effect for phosphorylated CREB^{D,4}, increasing the affinity by a factor of 2. In the presence of 5 mM Mg²⁺, phosphorylation at 1, 2, 3 or 4 sites in the CK cassette had a weaker inhibitory effect on DNA binding than in its absence (Fig. S6). Phosphorylated residues are able to bind divalent cations such as calcium and magnesium.^{30,31} The increased affinity observed for CKII-phosphorylated CREB constructs in the presence of 5 mM magnesium (i.e. reduction in ability to inhibit DNA binding) is likely due to binding of Mg²⁺ to the phosphorylated residues to neutralize their charge. Overall, by reducing the DNA binding affinity of non-phosphorylated CREB and increasing the binding affinity of the CK-phosphorylated protein, magnesium has the effect of dampening the dynamic range of the phosphorylation-dependent regulatory rheostat.

Phosphorylation inhibits DNA binding by an intramolecular mechanism

The NMR experiments showing the interaction between the KID and bZip domains were performed with fragments of CREB consisting of the isolated V118D KID and bZip domains, whereas the affinity measurements were performed with full-length V118D CREB. To assess whether phosphorylation can inhibit DNA binding in *trans*, we measured the DNA binding affinity of the isolated bZip domain in the presence of either non-phosphorylated or multisite-phosphorylated V118D KID domain (p^{CK}KID). Measurements were performed in high magnesium buffer (5 mM MgCl₂). Compared to full length CREB (average K_d 9 nM), the DNA binding affinity of the isolated bZip domain is reduced (K_d ~49 nM, Table S1) and is not much affected when preincubated with an equimolar ratio of either KID (K_d ~38 nM) or p^{CK}KID (K_d ~32 nM) (Fig. S7A). The inability of the fully phosphorylated p^{CK}KID peptide to inhibit DNA binding in *trans* provides strong evidence that, for full-length CREB, binding to the CRE is inhibited by intramolecular interactions between the phosphorylated CK cassette and residues within the basic region of the same CREB dimer.

Effects of phosphorylation-mimicking mutations

In some systems the regulatory effects of phosphorylation can be mimicked by replacing Ser or Thr with Glu or Asp.^{32,33} The K_d measured in 5 mM MgCl₂ for a CREB construct where residues 108, 111, 114, 117, and 119 were mutated to Glu (CREB^{E,5}) was 41 ± 7 nM (Fig. S7B, Table S1), compared to 230 ± 40 nM for CREB^{D,4} under the same conditions. We

conclude that phosphorylation-mimicking acidic mutations are not able to replicate the effects observed upon phosphorylation.

Effects of removal of the Q2 domain

The Q2 domain of CREB is important for recruiting the basal transcriptional machinery through interactions with a component of the TF_{II}D complex. Deletion of the Q2 domain, but not Q1, reduces CREB residence time on chromatin without directly affecting its affinity for DNA.^{34,35} A naturally occurring CREB isoform that lacks the Q2 domain, htCREB, is highly expressed in human adult testis and has been suggested to function in spermatogenesis.³⁶ To facilitate *in vitro* phosphorylation of the CK cassette in htCREB, the V118D phosphorylation-enhancing mutation was introduced to create an htCREB^{D,4} construct which, aside from the Q2 domain deletion (residues 162–272), is identical to the CREB^{D,4} construct. The DNA binding affinity of phosphorylated and non-phosphorylated htCREB^{D,4} was measured in both low and high Mg²⁺ buffer. Without phosphorylation, both constructs bound DNA with comparable affinity (Table S1). In contrast, upon phosphorylation by CKII, the htCREB^{D,4} isoform bound DNA over 10 times more weakly than CREB^{D,4} ($K_d \sim 6000$ nM, compared to 520 nM for CREB^{D,4}, Table S1) in low Mg²⁺ buffer, which more closely approximates the concentration of free Mg²⁺ in the nucleus.³⁷ The greater inhibitory effect observed for the htCREB^{D,4} isoform, both in low and high Mg²⁺ buffer, could be due in part to the closer sequence proximity between the phosphorylated residues and the bZip DNA binding domain, or alternatively to Q2-specific interactions with bZip or Q1 that are abrogated in the Q2-truncated construct. The larger effect of phosphorylation on htCREB^{D,4} will likely render this isoform more responsive to regulation by phosphorylation-inducing mechanisms such as cell-cycle progression and genotoxic stress.

Discussion

Phosphorylation can modulate regulatory responses by multiple mechanisms, including changes in protein structure, localization, or binding affinity.³⁸ Functional modulation can occur through single or multiple phosphorylation events and proceed via different response modalities such as on/off switches or rheostat-like behavior.^{39,40} Transcriptional activation by CREB is regulated by several phosphorylation-dependent regulatory pathways, mediated by different kinases.^{24,41} The canonical activation pathway involves phosphorylation of a single residue, S133, which activates transcription by recruitment of the coactivators CBP or p300 through switch-like interactions with their KIX domains.^{21,42} In contrast, the inhibitory pathway mediated by the CK/ATM cassette, which results in decreased chromatin occupancy and impaired CRTC2 coactivator recruitment, requires multisite phosphorylation for maximal response.¹¹ The reduction in chromatin occupancy and coactivator binding appears to be correlated with the number of phosphoserine residues, suggesting regulation by a graded (rheostat-like) mechanism.¹¹

Here we show that multisite phosphorylation of the CK cassette directly inhibits CREB binding to cognate DNA. The extent of inhibition is directly proportional to the number of phosphoryl groups. Quantitative measurements of the binding affinity using fluorescence

anisotropy show that phosphorylation functions as a rheostat that reduces the binding affinity by about 0.5 kcal/mol with each successive phosphorylation event. Phosphorylation increases the negative charge on the KID domain, promoting interactions between the phosphorylated region and residues in the basic region of the bZip that are required for DNA binding. Binding of the phosphorylated KID peptide to the bZip construct does not appear to induce significant structuring of the intrinsically disordered KID domain; except for the phosphorylated residues themselves, only very small chemical shift changes are observed in the ^1H - ^{15}N HSQC spectrum of phosphorylated KID upon addition of bZip (Fig. 3, Fig. S4). Likewise, chemical shift changes of basic region cross peaks are also small (Fig. 4, Fig. S5), showing that the basic region remains disordered upon binding to phosphorylated KID. While binding of the isolated p^{CK}KID and bZip peptides is in fast exchange on the chemical shift time scale, the intramolecular (*cis*) interaction within full-length CREB is on the slow-intermediate time scale and appears to be tighter than binding in *trans*.

A graded response to phosphorylation has been observed previously for other regulatory proteins.^{26,27,43} In particular, multisite phosphorylation of the transcriptional activation domain of p53 enhances binding to the TAZ1, KIX, and TAZ2 domains of CBP/p300 in a graded manner²⁶ while successive phosphorylations of the transcription factor ETS-1 result in a graded decrease in DNA-binding.²⁷ The magnitude of the graded responses exhibited by p53, ETS-1, and the CREB CK cassette are remarkably similar (0.6, 0.4, and 0.5 kcal/mol/phosphoryl for p53, ETS-1, and CREB, respectively).

The interactions between the phosphorylated KID domain and the bZip DNA binding domain are clearly predicated on the increase in negative charge as phosphoryl groups are added. It is notable that the inhibitory effects of multisite phosphorylation could not be recapitulated using phospho-mimetic mutations, likely due to differences in the physiochemical properties of phosphate and carboxyl groups, including their size and charge density.^{44–46} The effects of increased concentrations of magnesium on the inhibition process also argues for a primarily electrostatic mechanism, and is consistent with screening of the negative charge of the multiple phosphoryl groups by interaction with this cation. In cells, magnesium exists both as free ions and bound to cellular components, including proteins and DNA. In mammalian cells, the steady state concentration of free Mg^{2+} in the nucleus is typically less than ~1 mM,^{37,47} conditions under which multisite phosphorylation of the CK cassette would be expected to strongly inhibit DNA binding. Changes in the concentration of magnesium or other divalent cations in response to cellular stimuli^{48–50} could result in differential regulation of CREB in response to multisite phosphorylation *in vivo*.

Differential regulation of CREB can also be modulated by the presence of alternative isoforms. The alternate isoform htCREB, which lacks the Q2 domain, exhibits a substantially stronger regulatory response to multisite phosphorylation than the full-length protein. This suggests that different isoforms of CREB could be regulated to a different extent by the same phosphorylation-dependent mechanism.

To facilitate efficient phosphorylation of the CK cassette for biophysical studies, we introduced the V118D mutation into rat CREB to mimic the sequence of *Drosophila* CREB2 (dCREB2), which appears to be constitutively phosphorylated *in vivo* by a CKII-like kinase.

¹⁴ The mammalian and *Drosophila* CREBs appear to be regulated by different mechanisms; DNA binding by dCREB2 appears to be activated primarily by dephosphorylation of the constitutive CK sites while phosphorylation of S231 (equivalent to S133 in mammalian CREB) appears to play only a secondary regulatory role.¹⁴ In contrast, phosphorylation of S133 is of central importance for activation of mammalian CREB, while phosphorylation of the CK cassette is tightly regulated and requires priming by phosphorylation at S111 in response to cellular growth signals or genotoxic stress.^{11–13} Phosphorylation of the CK cassette constitutes a versatile regulatory mechanism by which the cell can fine tune CREB activity in response to changes in cellular conditions, modulating its DNA-binding activity in a graded manner that is directly proportional to the number of phosphoryl groups. It is interesting to speculate that intermediate levels of phosphorylation may allow the cell to differentially regulate CREB-responsive genes, suppressing weak CREB promoters while continuing to activate genes with high affinity CREB binding sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic diagram of the domain organization of CREB showing glutamine rich domains (Q1 and Q2), kinase inducible domain (KID), and basic region leucine zipper DNA binding and dimerization domain (bZip). A sequence alignment of the CK/ATM cluster (residues 108–122 of human CREB) of human and rat CREB and *Drosophila* dCREB2 is shown. Sites of phosphorylation in the CK cassette are in red. The PKA phosphorylation site at Ser133 is shown in green and the ATM site at Ser121 is in blue. The sequence of the engineered CREB construct used in this work (CREB^{D,4}) is also shown, with the V118D mutation indicated by an arrow.

Figure 2.

(A) Region of the ^1H - ^{15}N HSQC spectra of non-phosphorylated V118D KID (black) and CKII-phosphorylated V118D KID (p^{CK}KID, red). The two cross peaks observed for pS117 arise from incomplete phosphorylation of T119 (pS117a - T119 not phosphorylated labeled in purple; pS117b - T119 phosphorylated, labeled in red). Assignments of non-phosphorylated and fully-phosphorylated KID are shown with black labels, assignments for partially phosphorylated species are indicated using the color code of Fig. S1B. (B) Region of the ^1H - ^{15}N HSQC spectrum of CKII-phosphorylated full-length CREB^{D4} in complex with unlabeled CRE DNA. T119 was fully phosphorylated in the full-length CREB sample.

Figure 3.

(A) Region of the ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled p $^{\text{CK}}$ KID before (black) and after addition of unlabeled bZip (red) to a mole ratio of 1:1.125 p $^{\text{CK}}$ KID:bZip dimer. (B) Region of the ^1H - ^{15}N HSQC spectrum of non-phosphorylated ^{15}N -labeled KID before (black) and after addition of unlabeled bZip (red). The mole ratio is the same as in panel A. (C) Weighted average chemical shift changes for p $^{\text{CK}}$ KID residues resulting from addition of bZip dimer at 1:1.125 mole ratio. Red bar indicates the CK cassette. (D) Weighted average chemical shift changes for non-phosphorylated KID resulting from addition of bZip dimer at 1:1.125 mole ratio. Proline residues are indicated by a P below the x-axis. Black circles at the x-axis indicate residues that could not be assigned.

Figure 4.

(A, B) Regions of the ^1H - ^{15}N HSQC spectra of ^{15}N -labeled bZip before (black and gray) and after addition of unlabeled p^{CK}KID (red and purple), to a mole ratio of 2:1 p^{CK}KID:bZip dimer. (C, D) Regions of the ^1H - ^{15}N HSQC spectra of ^{15}N -labeled bZip before (black and gray) and after addition of non-phosphorylated V118D KID (red and purple) to a mole ratio of 2:1 KID:bZip dimer. “Rsc” denotes (aliased) arginine side chain resonances, “N293sc” denotes one of the Asn293 side chain $\text{N}^{\delta}\text{H}$ resonances. (E) Weighted average chemical shift changes for basic region cross peaks resulting from addition of excess p^{CK}KID (2:1 mole ratio relative to bZip dimer). (F) Weighted average chemical shift changes for basic region cross peaks resulting from addition of excess non-phosphorylated KID (2:1 mole ratio relative to bZip dimer).

Figure 5.

(A) Region of the ^1H - ^{15}N HSQC spectra of CKII-phosphorylated full-length CREB^{D,4} before (red) and after addition of equimolar unlabeled CRE DNA (black). Assigned backbone amide cross peaks of pSer and pThr residues of the KID domain are indicated. (B) Increase in intensity of pSer and pThr cross peaks upon addition of CRE DNA. The bar graph shows the ratio of cross peak heights ($I_{\text{CREB_DNA}}/I_{\text{CREB_free}}$) scaled relative to G6 and G13, which are unperturbed by DNA binding ($I_{\text{CREB_DNA}}/I_{\text{CREB_free}} = 1$). The asterisk indicates overlapped peaks.

Figure 6.

Measurement of DNA binding affinity using fluorescence anisotropy. (A) Changes in fluorescence anisotropy of Cy5-labeled half-site CRE DNA upon titration with V118D CREB constructs with 0 to 4 phosphoryl groups in the CK cassette (blue to red curves). The titrations were performed in low Mg^{2+} buffer ($< \sim 0.7$ mM residual Mg^{2+} from dilution of the phosphorylation buffer). (B) K_d values measured for constructs CREB^{D,0} (blue), CREB^{D,1} (green), CREB^{D,2} (yellow), CREB^{D,3} (orange), and CREB^{D,4} (red). (Inset) Plot showing binding free energies of CREB constructs versus number of phosphorylated residues in the CK cassette.