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Calmodulin Kinase II Attenuation of Gene Transcription by Preventing cAMP Response Element-binding Protein (CREB) Dimerization and Binding of the CREB-binding Protein*

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Calmodulin Kinase II (CamKII) inhibits the transcription of many CRE-dependent genes, but the mechanism of dominant transcriptional inhibition is unknown. Here we show that phosphorylation of serine 142 in CREB by CamKII leads to dissociation of the CREB dimer without impeding DNA binding capacity. CamKII-modified CREB binds to DNA efficiently as a monomer; however, monomeric CREB is unable to recruit the CREB-binding protein (CBP) even when phosphorylated at serine 133. Thus, CamKII confers a dominant inhibitory effect on transcription by preventing dimerization of CREB, and this mechanism may account for the attenuation of gene expression.

It is well established that both Ca^{2+}-calmodulin and cAMP-PKA[1] signals are involved in neuronal gene expression that underlie plasticity (1–7). However, the mechanism by which multiple signals coordinate neuronal transcription is not well understood. Both calcium and cAMP can activate genes containing conserved CREs (8–14). CRE-binding proteins including CREB-1 and ATF1 are substrates for both PKA and calcium-dependent kinases such as nuclear CamKIV (15–19). These kinases activate genes through their ability to phosphorylate CREB-1 at serine 133, a modification that is known to increase the affinity of CREB-binding protein and transcription (20–24).

However, elevation of calcium also stimulates Ca^{2+}/calmodulin-dependent protein kinase type II, which is by far the most abundant kinase in the neuron (25, 26). CamKII inhibits transcription of many CRE-dependent reporter genes, and CamKII inhibition of transcription dominates stimulatory effects of PKA (27) or CamKIV (15–19). CamKII modifies CREB at both serine 133 and 142 (14, 16–19). Because modification at serine 133 is stimulatory, inhibition is thought to involve serine 142 although cooperative interactions with serine 133 have not been excluded (15–19). However, the mechanism by which CamKII inhibits CRE-dependent transcription and attenuates the CamKIV or PKA response is not understood.

MATERIALS AND METHODS

Plasmids and Transfection—The pSomCAT (pss70CAT (9)) and the DynCAT reporter genes have been previously reported (27–29). CAT refers to the bacterial chloramphenicol acetyl transferase gene. Mammalian expression vectors for CREB proteins were previously constructed by modifying a vector containing a RSV promoter and a histidine fusion of the entire rat CREB (pET22b(+)His_6-CREB) (29, 43). Mutant forms of CREB lacking serine phosphorylation sites were generated by overlap polymerase chain reaction using primers which contained a T→G point mutation in the first residue of the serine codon (TCT), altering it to an alanine codon (GCT). All plasmids were verified by sequencing. Transfections utilized either human neuroblastoma (SK-N-MC) cells or African green monkey kidney cells (CV-1) cultured according to supplier's (ATCC) instructions. Transient transfection protocols utilized calcium phosphate as has been described previously (29, 43). Cells in each plate received 5.0 μg of CAT-reporter plasmid and 5.0 μg of MCV-PKA (28), RSV-CaMKII-(1–290) (15), or RSV-CaMKIV-(1–313) (15).

Protein Expression, Phosphorylation, and Purification—The pET22b(+) vectors that express His_6-CREB, His_6-CREB-S133A, His_6-CREB-S142A, or His_6-CREB-S133A/S142A fusion proteins were transformed into *Escherichia coli* strain BL21(DE3), and protein was expressed and purified as described previously (29). His_6-CREB in the text is referred to as CREB. CREB or mutant CREB was incubated with CaMKII (New England BioLabs) at a ratio of 1 unit of enzyme/pmol protein at 37 °C for 1 h in the presence of 1.0 mM ATP, 2.0 mM $CaCl_2$, and 2.4 μM calmodulin in reaction buffer (20.0 mM Tris, pH 7.5, 10.0 mM $MgCl_2$, 0.50 mM dithiothreitol, 0.10 mM Na_2EDTA). Phosphorylation of CREB or its mutants by PKA has been previously described (29, 43). The efficiency of CREB phosphorylation was quantified by phosphorimaging.

Gel Mobility Shift—Gel mobility assays have been previously described (29, 43, 44). The CRE binding templates were a 27-bp somatostatin CRE (5′-AGAGATTGCCTGAC-GTCAGAGAGCTAG-3′) and a 23-bp dynorphin CRE3 (5′-GTGGCTGCTGCG-TCAGAGCATGA-3′). Added protein ranged from 20.0 to 150.0 pmol and was incubated with 25.0 pmol of ^{32}P-endlabeled probe.

CBP Binding Assay—A carboxyl-terminal truncation of the CBP fusion protein (His_6-CBP-(1–682)) was expressed in *E. coli* strain BL21(DE3) using a His_6-mCBP-(1–682)pET15b vector (29). The bacterial extract was prepared as described (29, 43) and was stored at −70 °C. CBP binding was analyzed on immobilized CREB/DNA complexes. DynCRE3 oligonucleotides were synthesized with a biotin label (5′A**B**TA**B**TGTGGCTGCTG-CGTCAGAGCATGA-3′) (Mayo core facility) and bound to streptavidin-agarose columns. DNA was added to the column and incubated overnight at 4 °C with gentle rocking to allow maximal DNA binding, after which the beads were washed extensively with binding buffer to repack the column. CREB·DNA complexes were formed by the addition of native or mutant CREB to the immobilized DNA in the column. For all experiments, CREB was added to obtain a constant subsaturated protein/DNA ratio (between 0.3 and 0.4) to ensure complete binding of all dimeric or monomeric CREB. Finally, CBP was added at a ratio of 1 CBP molecule per CREB·DNA complex and incubated in a similar manner as for CREB. CBP or CREB proteins

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; CRE, cAMP responsive enhancer; CREB-1, cAMP response element-binding protein; CamKIV, calmodulin-dependent kinase type IV; CBP, CREB-binding protein; CamKII, Ca^{2+}/calmodulin-dependent protein kinase type II; CAT, chloramphenicol acetyl transferase; Som, somatostatin; Dyn, prodynorphin; RSV, Rous sarcoma virus.

were eluted in 20.0 mM Tris, 1.0 M KCl, 1.0 mM dithiothreitol, 1.0 mM $MgCl_2$, 0.5 mM EDTA, 10.0% glycerol, pH 6.8. Separated proteins were transferred to nitrocellulose membrane (Micron Separations Inc) and detected with antibodies specific for CREB or CBP (1:500 dilution of a rabbit anti-CBP polyclonal antibody, CBP (A22)) (Santa Cruz Biotechnology). The relative amount of His_6-CREB and His_6-CBP-(1–682) were evaluated by antibody detection (29) of an equal volume of eluate obtained under identical column conditions. Each experiment was repeated at least four times.

Sedimentation Equilibrium—Sedimentation equilibrium measurements were performed on an Optima XL-A equipped with UV-vis detection system (Beckman Instruments) as described previously (29, 45). For DNA/protein complexes, His_6-CREB and P-His_6-CREB were incubated with 1.0–2.5 μM somatostatin CRE (5′-GCCTCCTTGGCTGACGTCAGAGAGAG-3′) or dynorphin CRE3 (5′-GTGGCTGCTGCGTCAGAGCATGA-3′) that was endlabeled with fluorescein as described previously (29). Complexes were formed after incubation of protein with DNA overnight at room temperature. Samples were analyzed only using double sector cells to increase the column length. Each sample was analyzed at multiple rotor speeds (between 8,000 and 20,000 rpm) and at multiple concentrations. Experiments were carried out at 20 °C in an ANTi60 rotor until equilibrium was achieved. Equilibrium distributions were detected by scanning the absorbance across the cell at 494 nm, the maximum absorbance wavelength for fluorescein. The use of fluorescein tag on the DNA allows analysis of DNA/protein complexes without interference from free protein or DNA as long as the DNA is fully bound. Neither His_6-CREB nor P-His_6-CREB absorb at 494 mm. Boundaries were analyzed as described above using relevant partial specific volumes. The partial specific volume (ν_o) of the fluorescein-labeled CRE-containing oligonucleotides were calculated as described previously (29, 45). The ν_o of the DNA/protein complex was 0.695 ml/g for a His_6-CREB dimer and 0.679 ml/g for a His_6-CREB monomer. For fitting, data from multiple rotor speeds and multiple concentrations were fit simultaneously to obtain a best value using a nonlinear Levenberg-Marquardt fitting routine incorporated into a macro using Kaleidograph (29) or using NONLIN (46). Fitting from both routines yielded similar results. Molecular weights for one or two components were obtained from the best fit of the data using nonlinear Levenberg-Marquardt fitting routine (47) according to the following,

$$A(r) = A(r_b)e^{\omega^2 M(1-\nu\eta_0)(r^2-r_a^2)/RT} \quad \text{(Eq. 1)}$$

where A is the absorbance, r_a is the distance of meniscus to the axis of the rotor, r is the distance of each point on the boundary to the rotor axis, ω is the angular speed, ν_o is the partial specific volume of the component, and M is the molecular weight of the component, which is obtained from the fitting. Under subsaturating conditions, fitting was performed assuming a two component system in which one component was fixed to be the free DNA.

RESULTS

CamKII inhibits transcription of many CRE-dependent reporter genes, and inhibition is independent of the CRE symmetry or CRE number (Fig. 1). In contrast to either CamKIV or PKA, constitutively active CamKII represses expression of CRE-containing CAT reporter constructs when co-transfected in mammalian cells (Fig. 1*A*). CamKII mediates the dominant inhibitory effect because overexpression of an inactive form of CamKII abrogates inhibition of either the SomCAT or DynCAT reporter genes (data not shown). SomCAT and DynCAT expression are inhibited by co-expression of CamKII with either CamKIV (not shown) or PKA (Fig. 1A), to the same extent as CamKII alone (Fig. 1*A*). Therefore, CamKII produces a dominant inhibitory effect that overcomes stimulation by either CamKIV or PKA (Fig. 1*A*).

CREB-1 is the major factor that mediates transcriptional regulation of both somatostatin (27) and prodynorphin by PKA (28). For both Dyn (Fig. 1*B*) and Som (Fig. 1*C*) constructs, we find that the inhibitory effect of CamKII is mediated by CREB and occurs at CRE elements required for stimulation by CamKIV or PKA. CRE-3 is the major site for CREB-dependent regulation of prodynorphin (28). In the presence of CamKII, DynCAT expression is inhibited relative to control (Fig. 1*B*). However, deletion or point mutations that abolish CREB-1 binding at the CRE-3 of prodynorphin (28) also relieve CamKII-dependent inhibition (Fig. 1*B*) relative to control. A mutant CREB (CREB-K304E (28)) that cannot bind to the CRE but is a substrate for CamKII phosphorylation relieves CamKII-dependent inhibition of either SomCAT (Fig. 1*C*) or DynCAT (Fig. 1*D*) promoters. Thus, gene activation by either CamKIV or PKA and inhibition by CamKII appear to involve modification of the same CREB·CRE complex.

CamKII modifies CREB-1 at two positions, serine 142 and serine 133 (Fig. 2*A*). In CV-1 cells, transcription of SomCAT is inhibited by expression of CamKII (Fig. 2*B*). To determine the

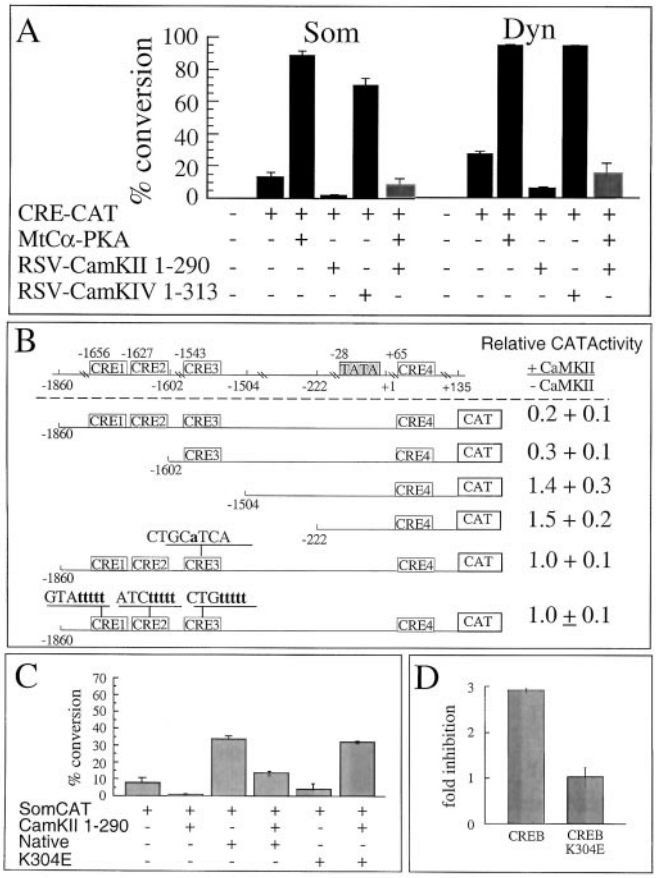


FIG. 1. **CamKII confers a dominant inhibitory effect on CREB-dependent gene expression.** *A,* effects of kinases on the transcription of CRE-containing genes. *Som* represents the promoter region of the somatostatin gene and contains only one symmetric CRE site. *Dyn* represents the promoter region of the rat dynorphin gene and contains four asymmetric (non-palindromic) CRE sites. In each experiment, CV-1 cells were transfected with 5 μg of pss70CAT (*Som*) or 2.0pCAT (*Dyn*) (26), 5 μg of MT-PKA (catalytic subunit), RSV-CaMKIV-(1–313), and RSV-CaMKII-(1–290), as indicated in CV-1 cells. An aliquot of 5 μg of each indicated plasmid was used. The (+) indicates the presence of the transfected plasmid whereas (−) indicates absence. *B,* schematic of dynorphin gene promoter (*top*) and dynorphin-CAT reporter genes (22). Relative activity is calculated by the ratio of CAT activity in the presence of CamKII to CAT activity in the absence of CamKII. Transfection conditions are as in *A*. *C,* the inhibitory effect of CaMKII on somatostatin activation depends on CREB binding to the CRE. Transfections are as in *A*; 5 μg of RSV-expression plasmids for CREB or K304E were utilized. Native refers to native CREB-1 protein; K304E refers to a CREB-1 mutant that contains a lysine to glutamic acid change and cannot bind to the CRE (26). *D,* CamKII-mediated repression of the rat prodynorphin promoter requires CREB binding. CREB is native CREB; K304E is CREB containing a lysine to glutamic acid base change in CREB. Fold inhibition is the ratio of CREB inhibition in the presence of CamKII to the absence of CamKII. Transfections are as in C; 5 μg of RSV-expression plasmids for CREB or K304E were co-transfected with CamKII-(1–290) and 2.0pCAT (*Dyn*). When CREB cannot bind, CamKII relieves inhibition of expression driven by the rat prodynorphin promoter.

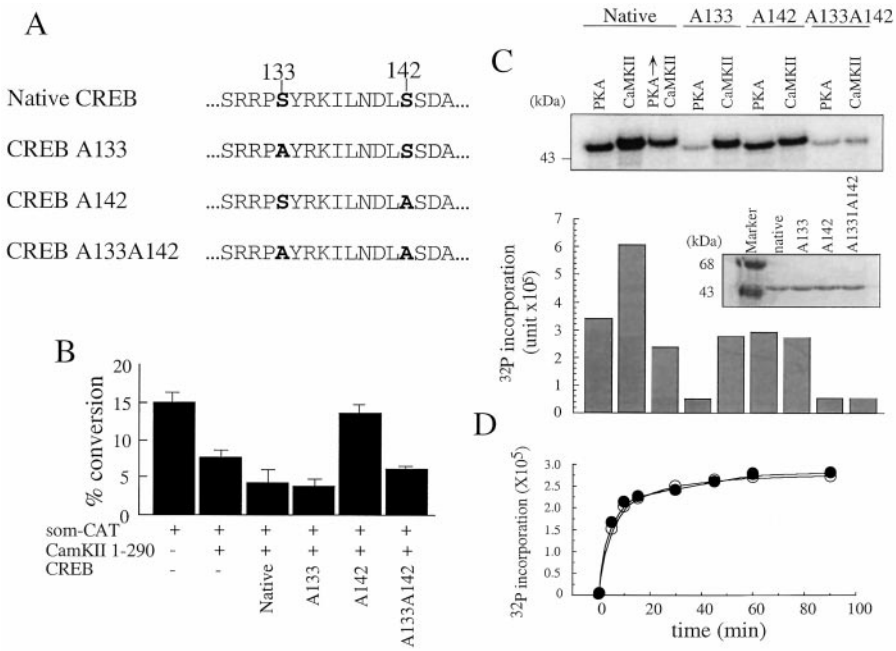
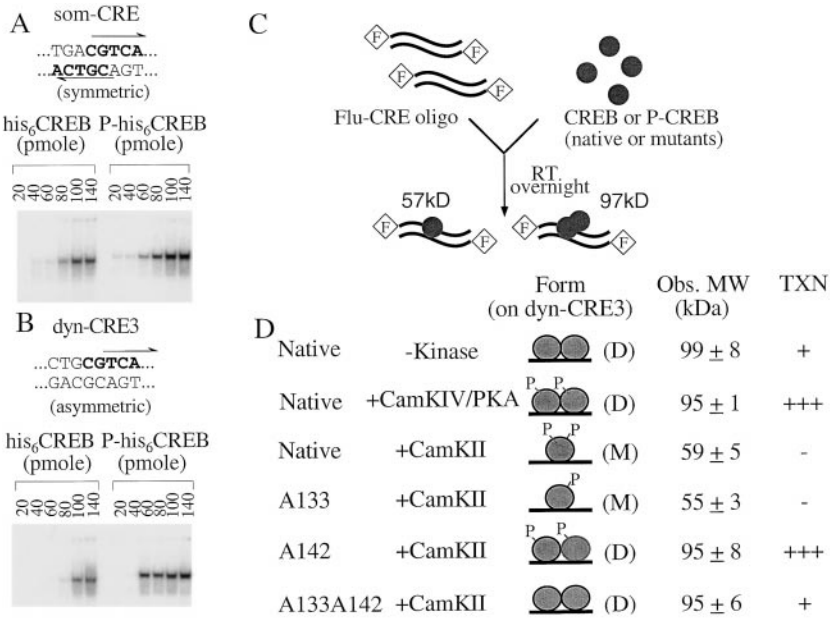


FIG. 2. **The inhibitory effect of CamKII requires phosphorylation of CREB at serine 142.** *A,* schematic diagram of CREB serine to alanine mutants. *Native* is CREB-1; serine to alanine changes at positions 133 (*A133*), 142 (*A142*) or both (*A133A142*) are indicated. *B*, effect of CamKII on SomCAT expression mediated by the native or mutant CREB proteins from *A*. Native or mutant CREB expression vector (5 μg) was cotransfected in CV-1 cells with CaMKII expression vector (5 μg) and somatostatin-CAT reporter plasmid (5 μg). In the absence of kinase, S142A and S133A/S142A had activities similar to native CREB whereas S133A displayed lower activity (6% conversion). In the presence of CamKII, mutation of serine to alanine at 142 relieves CamKII inhibition. The inhibitory effect of CaMKII on gene expression of CRE-dependent genes requires phosphorylation of CREB at serine 142. *C,* CamKII phosphorylates serines 133 and 142 equally. Purified native or mutant CREB (100 pmol) was phosphorylated *in vitro* by either PKA or CamKII using [γ-^{32}P]ATP. Some residual binding of labeled nucleotides can be observed, and these can be subtracted from the total counts. PKA→CamKII indicates that or native CREB was phosphorylated sequentially by PKA using unlabeled ATP followed by CamKII using [γ-^{32}P]ATP. *Top*, a representative autoradiogram of phosphorylated proteins after separation on 10% denaturing polyacrylamide gel; *bottom*, the incorporation of ^{32}P was quantified by phosphorimager; *inset,* the protein substrate used in the labeling experiment was stained with Coomassie Blue to confirm equivalent loading of native and mutant proteins. *D*, CamKII phosphorylates each site at equal rates. CREB-S133A (*open circles*, 545 pmol) or CREB-S142A (*filled circles*, 545 pmol) were phosphorylated by CamKII in the presence of [γ-^{32}P]ATP. Products were separated on 10% denaturing polyacrylamide gel and incorporation of ^{32}P was quantified by phosphorimaging.

relationship between CREB phosphorylation and CaMKII-mediated inhibition, we constructed and expressed native and three mutant forms of CREB. Each mutant contains a serine to an alanine change at either or both phosphorylation sites (Fig. 2*A*). In the absence of kinase, native CREB, S142A, and S142A/S133A displayed similar activity (Fig. 2*A, Native CREB,* −Kinase). In the presence of CamKII, SomCAT expression is inhibited by expression of either native CREB or the S133A mutant, both of which can be phosphorylated at serine 142 (Fig. 2*B, Native versus A133*). Relative to native CREB, inhibition of SomCAT is relieved in the presence of the S142A and S133A/S142A mutants, neither of which can be phosphorylated at serine 142 (Fig. 2*B*, *Native versus A142* and *A133A142*). Because inhibition is not significantly different between native CREB and the CREB-S133A mutant, the data indicate that inhibition is not a cooperative effect of modification at both sites. Rather, the inhibitory effect of CamKII requires only CREB phosphorylation at Ser-142. Interestingly, we never see expression of SomCAT with S142A in the presence of kinase equal to native CREB in the presence of the kinase. However, there is endogenous CREB in the cells. Therefore, we expect that in some cases the S142A mutant will dimerize with a native CREB molecule that contains an intact 142 site hence lowering expression of the reporter.

We next examined why phosphorylation of CREB at 142 is inhibitory. Phosphorylation at serine 142 by CamKII might be preferred and might negatively influence the extent of phosphorylation at serine 133. In this case, the inability to efficiently modify CREB at 133 may explain the inhibitory effect. To test whether the serine 142 site is preferred, we evaluated the efficiency of CREB phosphorylation by CamKII at each site. Native or mutant CREB was incubated with CamKII or PKA in the presence of [^{32}P]ATP and the extent of phosphorylation was measured *in vitro* (Fig. 2*C*). We find that CaMKII alone efficiently modifies native CREB at both 133 and 142. CREB contains twice as much incorporated label after CamKII phosphorylation as after PKA phosphorylation. Modification of either S133A or S142A by CamKII occurs equally and about half as well as for the native CREB (Fig. 2*C*). In all cases, mutant CREB proteins are equally expressed and are appropriate substrates for phosphorylation. Mutant S142A cannot be phosphorylated on serine 142 and is phosphorylated to the same extent by CamKII as is native CREB by PKA. Additionally, S133A is not a substrate for PKA, and the double mutant is not a substrate for PKA or CamKII (Fig. 2*C*). Finally, CREB modification at the inhibitory site 142 occurs at an equal rate as for the stimulatory site at 133 (Fig. 2*D*). Thus, inhibition by CamKII does not occur because serine 133 is slowly modified. We conclude that CamKII displays no site preference and modifies either the 133 or the 142 site to the same extent.

Using these mutants, we also confirmed that CamKII modification of CREB at these two sites is not cooperative. Phosphorylation of CREB by PKA at serine 133 does not prevent or influence modification at serine 142 by CamKII (Fig. 2*C*). This is evident from the fact that incorporated label at site 142 in native CREB by CamKII is roughly the same before or after phosphorylation at 133. This means that CamKII can fully modify CREB even after phosphorylation by PKA or CamKIV (Fig. 2*C*). Similarly, mixing CamKII-modified S133A and native CREB does not prevent efficient phosphorylation at serine 133 (not shown). Thus, CamKII efficiently phosphorylates either site independent of modification at the other. We conclude



E

Samples	Predicted MW (kDa)	
	somatCRE	dynCRE3
free DNA	18	17
monomer/DNA	59	57
dimer/DNA	99	97

F

P/D ratio	Observed MW (kDa)							
	som-CRE			dyn-CRE3				
	Native	P-Native	P-A133	Native	P-Native	P-A133	P-A142	P-A133 A142
0	19 ± 2	19 ± 2	19 ± 2	17 ± 2	17 ± 2	17 ± 2	17 ± 2	17 ± 2
0.5	---	58 ± 3	56 ± 8	85 ± 6	53 ± 2	54 ± 5	---	---
0.6	104 ± 8	---	---	---	---	---	---	---
1.0	104 ± 5	60 ± 4	59 ± 5	89 ± 3	59 ± 5	55 ± 3	95 ± 8	95 ± 6
2.0	105 ± 4	53 ± 1	62 ± 4	93 ± 4	58 ± 2	59 ± 6	---	---

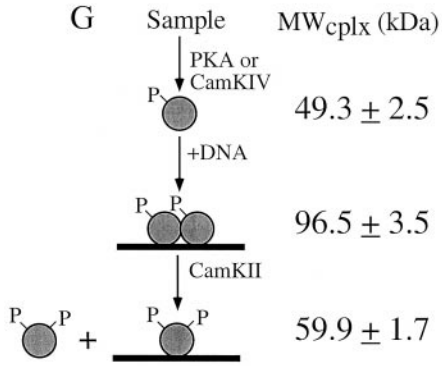


FIG. 3. **Phosphorylation by CamKII at serine 142 prevents CREB dimerization.** *A* and *B,* gel shift analysis of CREB·DNA complexes. The binding of CREB to Som-CRE (*A*) or Dyn-CRE3 (*B*) is not significantly influenced by phosphorylation by CamKII. Binding affinity is monitored by gel mobility shift assay using either native CREB (*his$_6$CREB, left*) or CREB phosphorylated by CamKII (*P-his$_6$CREB, right*) complexed with the indicated radiolabeled CRE templates (25 pmol). Increasing amounts of added CREB are indicated in pmols. *C,* experimental design for sedimentation equilibrium experiment using fluorescein-labeled DNA templates. DNA templates were identical to those used in gel shift analysis with the exception of a single fluorescein residue located at the 5′-end of the template. CREB·CRE complexes were incubated at room temperature overnight before analysis. The predicted molecular masses for monomer and dimer complexes are indicated. *D,* sedimentation equilibrium analysis of native and mutant CREB·DNA complexes before and after phosphorylation with various kinases. The boundaries at equilibrium were detected by absorbance at 494 nm, the λ_{max} of fluorescein. The molecular weights were determined by the best fit to Equation 1. Native or mutant CREB in the complex is indicated. The DNA template reported is the 23-base pair DynCRE3 oligonucleotide although similar results were observed using the somatostatin CRE (not shown). Molecular mass of CREB·DNA complex in the absence of phosphorylation (*−Kinase*); molecular mass of a CREB·DNA complex after modification by either PKA or CamKIV in independent experiments (*+CamKIV/PKA*); and CREB·DNA complexes in the presence of calmodulin kinase II (*+CamKII*) are shown. Form indicates a bound monomer (*M*) or dimer (*D*). The *Obs. MW* (*kDa*) is the measured molecular mass of the complex by the sedimentation equilibrium analysis. *Txn* indicates the relative transcriptional activity supported by each mutant. *Txn* correlates directly with CREB dimerization. *E,* predicted molecular masses for DNA 27-bp somCRE or the 23-bp DynCRE3 templates

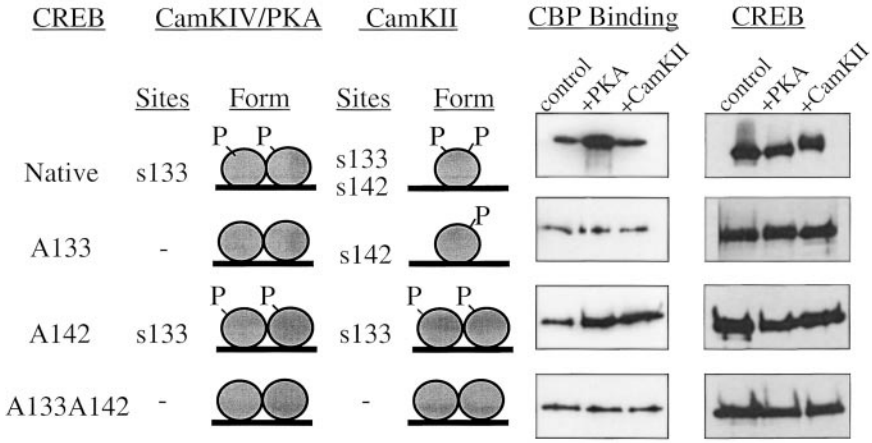


FIG. 4. **CaMKII prevents of CREB dimerization and CBP binding to CREB·DNA complex.** *A,* CamKII phosphorylation causes loss of dimerization and prevents CREB-CBP interaction. Sites for PKA or CamKII modification of native or mutant CREB are indicated. *Form* indicates the monomer or dimer state of CREB after modification as measured by sedimentation equilibrium by either PKA or CamKIV (*CamKIV/PKA*) or CamKII. CBP binding is the amount of CBP-(1–682) bound to a CREB·dynCRE3 complex immobilized on a streptavidin column. CBP-(1–682) was detected using specific antibodies after elution from the column with a high salt wash. In this experiment, the DynCRE3 oligonucleotide was modified on the 5′-end with biotin to allow association with the streptavidin matrix. CBP binding was evaluated using three different CREB substrate complexes immobilized on independent columns. CBP substrates are defined as follows: *control* is a CREB·DNA complex in the absence of phosphorylation; *+PKA* indicates a CREB·DNA complex in the presence of phosphorylation by PKA; *+CamKII* indicates a CREB·DNA complex in the presence of phosphorylation by CamKII. CREB indicates the amount of CREB that was dissociated from each column. Each column contained the same amount of CREB. This was confirmed by evaluating the amount of CREB eluted in a high salt wash at the end of the experiment (*CREB*).

that CamKII-mediated inhibition can prevent stimulation by PKA or CamKIV, but PKA or CamKIV modification does not prevent a subsequent inhibitory modification at 142. Phosphorylation of CREB at serine 142 by CamKII is necessary and sufficient for the dominant inhibitory effect of CamKII on transcription. However, differential phosphorylation does not explain inhibition.

CamKII-modified CREB might inhibit transcription by reducing binding of CREB at the CRE site. We find, however, that CamKII does not decrease and, in fact, modestly increases binding of purified CREB to either the SomCAT (Fig. 3*A*) or DynCAT CREs (Fig. 3*B*). Because binding of CREB is required for activity of the somatostatin promoter, it is unlikely that transcriptional attenuation by CamKII is caused by increasing DNA binding activity there.

Instead, we find that CamKII-mediated phosphorylation of serine 142 attenuates dimerization of full-length CREB (Fig. 3, *C* and *D*). The self-association state of CREB is measured by sedimentation equilibrium using fluorescein-labeled DNA templates (Fig. 3*C* and Ref. 29). The use of fluorescein labels allows detection of the protein/DNA complex using visible light without interference from the free protein (29). Under conditions of transcriptional activity, native CREB is a dimer and activity is enhanced by phosphorylation at serine 133 (Fig. 3*D*, *Native −kinase* and *+CamKIV/PKA*). Transcription is also supported by both CamKII-modified S142A mutant and the S133A/S142A double mutant (Fig. 3*D*). Neither mutant can be phosphorylated at serine 142 and both are dimers on DNA. In contrast, transcription is inhibited when CamKII modifies the native or S133A mutant, both of which are phosphorylated at position 142. It has been clearly demonstrated that unphosphorylated CREB or PKA-modified CREB binds to DNA as a dimer (9, 29, 30, 31). However, after modification by CamKII, CREB binds DNA efficiently as a monomer, but monomer binding is not associated with transcriptional stimulation (Figs. 3, *D–F*, 1*A,* and 2*B*). When serine 142 is phosphorylated, the monomer-dimer equilibrium is shifted far toward monomer because dimer assembly is prevented even under high concentrations of CREB (Fig. 3, *E* and *F*). For the native protein, transcription is inhibited and dimerization is prevented even when serine 133 is modified (Fig. 3*D*, *Native +CamKII*). Modification of CREB at serine 142 prevents CREB dimer assembly at the CRE. If modification at serine 142 also causes previously bound dimers to dissociate, then loss of dimerization might explain the dominant inhibitory effect of CamKII on transcription. Indeed, bound CREB dimers dissociate after CamKII modification before or after serine 133 modification by PKA (Fig. 3*G*). We conclude that CamKII inhibits transcription by preventing CREB dimerization on DNA.

It is well documented that CREB phosphorylation at serine 133 stimulates transcription by increasing the binding affinity of CBP (20–24, 32). In contrast, protein-protein association of a 59-amino acid KID domain peptide in CREB with the KIX domain peptide fragment in CBP is inhibited by phosphorylation at serine 142 (20). We, therefore, asked whether phosphorylation at 142 and loss of CREB dimerization inhibit transcription by weakening CBP binding to full-length CREB bound to DNA. Full-length native or mutant CREB was bound (ratio of 0.4) to immobilized biotin-labeled dynorphin CRE3. Purified CBP was added last to the CREB·DNA complex, and CBP binding was evaluated. CBP binds modestly to unphosphorylated CREB, and binding is significantly enhanced by PKA modification at serine 133 (Fig. 4). Under these conditions, CREB is a dimer (Fig. 4). In contrast, CBP binding is not enhanced on native CREB after CaMKII modification even though serine 133 is modified (Fig. 4*A*). CBP binding is also not

and their respective CREB·DNA complexes. *F,* molecular masses determined by sedimentation equilibrium analysis for native and mutant CREB complexes before (*native*) and after (*P−*) phosphorylation with CamKII. The complexes with an increasing range of protein/DNA (*P/D*) ratios were evaluated as in *C* and *D*. (—) denotes that the complex was not analyzed at the indicated P/D ratio. *G,* CamKII dissociates bound CREB dimers in the presence of CamKIV or PKA phosphorylation. CREB protein is modified by PKA or CamKIV and bound to DNA. The bound complex was then treated with CamKII. At each step, the resulting complexes were evaluated by sedimentation equilibrium analysis. The best fit for the molecular mass at each step is indicated.

enhanced for the S133A mutant after modification by CamKII. Under these conditions, native CREB and the S133A mutant are monomers (Fig. 4). In all CBP binding experiments, the amount of CREB substrate is similar (Fig. 4, *far right panel*). Thus, monomers of CREB efficiently bind to DNA when modified by CamKII, but CBP is unable to bind to monomeric CREB even when serine 133 is phosphorylated. In other words, phosphorylation at Ser-133 is necessary but not sufficient for recruiting CBP to the CREB·CRE complex. CREB dimerization is also required.

DISCUSSION

The mechanism by which CamKII inhibits CREB-dependent reporter activity is not well understood. We show here that phosphorylation of CREB at serine 142 prevents dimerization and uncouples CBP binding. Many studies have shown that CREB is a dimer in its unphosphorylated state and when modified by PKA (9, 29–31). Therefore, our results suggest a model in which CREB dimerization acts as a transcriptional switch that operates through phosphorylation and allows a distinct response depending on the kinase. The data reveal several features that may be important with respect to CREB interactions *in vivo*.

First, CamKII-induced loss of CBP association and CREB dimerization occurs when CREB is bound to DNA. Therefore, these events can directly lead to transcriptional inhibition. It had previously been shown that casein kinase II modification of a 59-amino acid region of the CREB KID domain inhibited protein-protein association with a KIX domain peptide of CBP (20). Phosphorylation at serine 142 likely disrupts interaction with a Tyr-650 that is an essential residue for the KID/KIK binding interface and the hydrophobic interface (20, 33). However, casein kinase II modifies at least four other sites within the KID domain. Heretofore, it was not known whether CBP dissociation occurred in the full-length CREB protein, whether dissociation occurred when CREB was bound to DNA, or whether dissociation involved CREB dimerization. Our results not only confirm that CREB modification at serine 142 is sufficient to account for the negative effects on transcription but also reveal that CREB dimerization is involved.

Second, CREB when modified by CamKII binds to DNA as a monomer. This was surprising because early studies using model leucine zipper and DNA binding domains suggested that dimerization was essential for CREB binding (34). Recently, however, detailed spectroscopic and kinetic studies using full-length proteins have revealed that CREB monomers sequentially assemble into a dimer on DNA (29, 35). Monomeric forms of CREB have also been reported to activate transcription (36). Sequential monomer binding is also observed for other bZip peptides including GCN4 and BLH (37). We have used sedimentation equilibrium to calculate the mass of the CREB·DNA complex. The analysis conclusively demonstrates that CamKII-modified CREB is a monomer when bound to DNA. This is in contrast to dimer binding of either unphosphorylated or PKA-modified CREB within the same experiment (Fig. 3*D*). Sedimentation equilibrium can be used reliably to evaluate monomer or dimer states in proteins because the calculated mass is independent of either the shape or the charge of the complex (38). Gel shift analysis also confirms that CREB remains bound to DNA when modified by CamKII. However, gel shift analysis cannot unambiguously confirm a monomer-dimer complex, especially when there has been a change in the phosphorylation state. It is well documented that proteins of very different mass can migrate at the same position on gels because of charge and shape effects (39, 40). This is because gel mobility in an electric field is proportional to the ratio of the charge to the frictional coefficient (37), both of which are altered by CREB phosphorylation (20). Indeed, the slower mobility of the CamKII-modified CREB·DNA complex relative to the native CREB·DNA complex implies that conformational change in CREB has occurred because the addition of two phosphate groups should enhance mobility if charge were the only factor. Although monomeric CREB can bind DNA, a CamKII-modified monomer cannot sustain productive interactions with CBP and the transcription machinery.

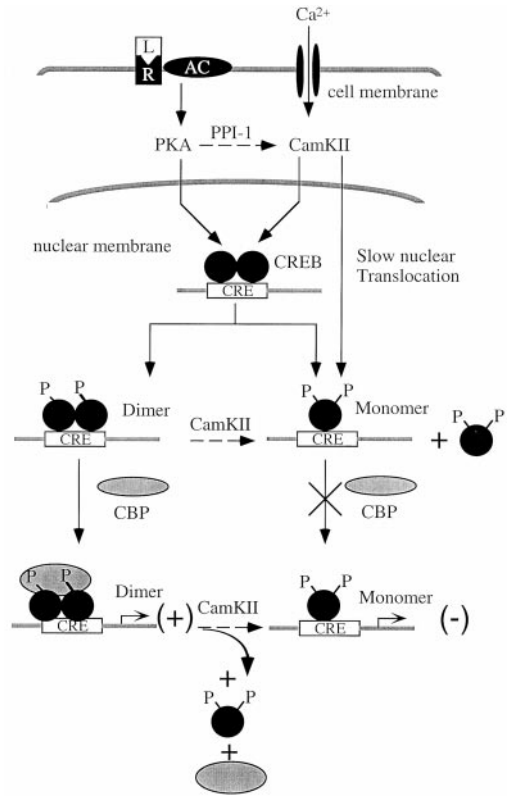


FIG. 5. **Switch model for CREB dimerization as an integrator of cAMP and Ca^{2+} pathways.** CREB dimerization allows CBP to bind and support transcription. Phosphorylation by CamKII attenuates transcription by preventing CREB dimerization and uncouples CBP binding at CRE sites. *AC*, adenylcyclase; *PPI-1*, protein phosphatase 1.

Finally, loss of CREB dimerization and CBP binding after phosphorylation by CamKII suggests a mechanism for the dominant negative effect on transcription at some genes as well as activation of others. CREB dimerization serves as a molecular switch that integrates calcium and cAMP signals and coordinates gene expression (Fig. 5). PKA or CamKIV can stimulate expression of CRE-dependent genes by CREB phosphorylation at serine 133. Under these conditions, CREB exists as a dimer on DNA and successfully attracts CBP to the target gene. However, in the presence of CamKII or other kinases that may modify CREB at serine 142, CREB dimerization is prevented and CREB is no longer able to compete for CBP binding (Fig. 5). Phosphorylation at serine 142 prevents CREB dimerization on DNA before or after binding. Because dimer dissociation can also occur before or after modification of Ser-133 by another kinase, phosphorylation of CREB at Ser-142 produces a dominant inhibitory effect. CamKII is highly concentrated in the dendrites where it constitutes the major protein of postsynaptic structures (25, 26, 41, 42). Because kinases often translocate slowly from synapse to nucleus, the timing may allow a window of opportunity for gene expression to occur before attenuation by CREB modification at serine 142. Thus, kinase modification of CREB at serine 142 can program gene expression by shifting the CREB dimer-monomer equilibrium.

Whereas CamKII widely inhibits expression of reporter

genes, a role for CamKII in transcriptional inhibition *in vivo* has not been clearly demonstrated. Therefore, regulation *in vivo* is likely to be more complex then is suggested by a simple reporter system. However, the ability of serine 142 phosphorylation to regulate CREB dimerization may also explain gene activation by CamKII. For gene activation, it is possible that modification at serine 142 and CREB dimer dissociation allow the formation of new heterodimers that restore affinity for CBP. Phosphorylation of CREB provides a mechanism for regulation of transcription because either the dimer or monomer can receive signals that influence the choice of partner. Differential phosphorylation may control selection of a new dimerization partner *in vivo* through a monomer intermediate. These effects are likely to play a role in response specificity and may broadly apply to other kinases. The ability to control CREB dimerization and CBP binding by phosphorylation at 142 provides a common mechanism by which signals from diverse pathways can be integrated to control diverse function and neuronal plasticity.

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Calmodulin Kinase II Attenuation of Gene Transcription by Preventing cAMP Response Element-binding Protein (CREB) Dimerization and Binding of the CREB-binding Protein

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