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Phosphorylation of CREB at Ser-133 Induces Complex Formation with CREB-Binding Protein via a Direct Mechanism

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We have characterized a phosphoserine binding domain in the coactivator CREB-binding protein (CBP) which interacts with the protein kinase A-phosphorylated, and hence activated, form of the cyclic AMP-responsive factor CREB. The CREB binding domain, referred to as KIX, is α helical and binds to an unstructured kinase-inducible domain in CREB following phosphorylation of CREB at Ser-133. Phospho-Ser-133 forms direct contacts with residues in KIX, and these contacts are further stabilized by hydrophobic residues in the kinase-inducible domain which flank phospho-Ser-133. Like the src homology 2 (SH2) domains which bind phosphotyrosine-containing peptides, phosphoserine 133 appears to coordinate with a single arginine residue (Arg-600) in KIX which is conserved in the CBP-related protein P300. Since mutagenesis of Arg-600 to Gln severely reduces CREB-CBP complex formation, our results demonstrate that, as in the case of tyrosine kinase pathways, signal transduction through serine/threonine kinase pathways may also require protein interaction motifs which are capable of recognizing phosphorylated amino acids.

A number of signaling pathways have been shown to regulate the expression of target genes by inducing the phosphorylation of specific transcription factors (9). The second messenger cyclic AMP (cAMP), for example, stimulates the expression of numerous genes through protein kinase A (PKA)-dependent phosphorylation of CREB at Ser-133 (7). Although phosphorylation may regulate such factors by enhancing their nuclear targeting or DNA binding activities, CREB belongs to a group of proteins whose transactivation potential is specifically affected (2, 8).

The CREB transactivation domain is bipartite, consisting of a kinase-inducible domain (KID) (amino acids [aa] 88 to 160) and a glutamine-rich constitutive activator termed Q2 (aa 161 to 283) which synergize to stimulate target gene expression in response to cAMP (2). When fused to a heterologous DNA binding domain such as that of GAL4, the KID can cooperate in *trans* not only with Q2 but also with a number of constitutive activators, including GAL4 and VP16. These observations suggest that the KID contains the minimal region which is both necessary and sufficient for PKA-regulated activity.

We and others have recently characterized a 265-kDa CREB-binding protein (CBP) which binds to CREB in a phosphorylation-dependent manner in vitro (4) and which is closely related to the E1A-associated protein P300 (6). CBP appears to be important for cAMP-responsive transcription, as illustrated by microinjection studies in which anti-CBP antiserum could block transcription from a cAMP-responsive promoter and by transfection studies in which a CBP expression vector could potentiate CREB activity in a phosphorylation-dependent manner (1, 11). It is not known, however, whether Ser-133 phosphorylation allows CREB to associate with CBP via an allosteric mechanism or a direct mechanism or whether the CREB-CBP interaction is in fact critical for cAMP-responsive transcription in vivo. Here we characterize a domain in CBP, termed KIX, which binds to the KID of CREB in a phospho-Ser-133-dependent manner. Our results suggest that KIX represents a novel motif which promotes protein-protein interactions in response to Ser/Thr kinase-mediated signals.

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MATERIALS AND METHODS

Binding assays. Glutathione *S*-transferase (GST) fusion proteins were expressed in pGEX-KT and purified as previously described (4). Recombinant CREB protein was labeled with [γ-^{32}P]ATP by using the purified catalytic subunit of PKA (PKA-Cα; a generous gift from S. Taylor) in a reaction volume of 25 μl [25 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.1 mg of bovine serum albumin (BSA) per ml, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml] for 1 h at 30°C. Free [^{32}P]ATP was removed by centrifugation through Centri-Sep columns (Princeton Separations, Inc.). Binding assays were performed in PC+100 buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 20% glycerol, 0.1 mg of BSA per ml, 1 mM phenylmethylsulfonyl fluoride). Labeled CREB was mixed with GST fusion proteins in 100-μl reaction volumes and incubated for 30 min at room temperature. Glutathione-Sepharose (20 μl of a 50% slurry) was added, and the mixture was incubated at room temperature with a rotator. Samples were washed three times with PC+100 buffer and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). ^{32}P-labeled CREB bands were quantified by PhosphorImager analysis.

Cross-linking assays. A CREB peptide consisting of aa 121 to 151 ($CREB_{121–151}$) was phosphorylated by PKA-Cα with either γ-^{35}S-ATP or [^{32}P]ATP in 20 mM Tris-HCl (pH 7.0)–10 mM $MgCl_2$–1 mM EDTA. For cross-linking, 6 pmol of phosphopeptide was preincubated in 20 μl of 20 mM sodium phosphate (pH 7.4)–100 mM KCl–2 mM $MgCl_2$–0.2 mM EDTA–0.05% Nonidet P-40–20% glycerol in the presence and in the absence of 1 mM K_2PtCl_4. After 10 min at room temperature, 1 nmol of the KIX S/B polypeptide (aa 553 to 679 of CBP) was added and allowed to react for 30 min at room temperature. The reaction was stopped by the addition of 1 volume of 2× nonreducing SDS loading buffer, and the resulting mixture was boiled for 1 min and electrophoresed on an SDS–15% polyacrylamide gel.

Circular dichroism studies. The $CREB_{88–160}$ peptide and KIX S/B were expressed in *Escherichia coli* with the pGEX-2T vector (Pharmacia) and purified on glutathione-Sepharose according to the supplier's instructions. After phosphorylation of $CREB_{88–160}$ with recombinant PKA-Cα (kindly provided by S. Taylor, University of California, San Diego), the samples were exchanged into 10 mM sodium phosphate (pH 7.4) and concentrated. For absorbance measurements, the samples were diluted to final concentrations of 5 μM peptide and 0.67 mM sodium phosphate, and the spectra were measured on an Aviv 60DS circular dichroism spectrometer with a 0.1-cm cuvette. Secondary structure was estimated from the data by using the PROSEC program.

Screening for mutant KIX cDNAs. Random mutations in the KIX domain of CBP were made by a modified PCR technique as described by Leung et al. (12). Following amplification, the PCR products were ligated into the pCRII-TA cloning vector (Invitrogen), transformed into One Shot cells, and plated. Bacterial colonies were then streaked onto isopropyl-β-D-thiogalactopyranoside (IPTG)-treated nitrocellulose filters and incubated overnight at 37°C. The colonies were then exposed to chloroform for 15 min and lysed in 100 mM Tris-HCl (pH 8.0)–150 mM NaCl–5 mM $MgCl_2$–1% SDS for 10 min on a rotator. After two washes in TNT buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20), the filters were incubated for 1 h in blocking buffer (1× phosphate-buffered saline [PBS], 5% nonfat dried milk, 1% BSA, 0.1% Triton X-100). The filters were then incubated with ^{32}P-labeled CREB for 2 h in buffer A (10 mM Tris [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% BSA, 5% nonfat dried milk, 0.1% Triton X-100, 1 mM EDTA, 5% glycerol) and subsequently washed twice for 30 min each time in buffer A. The filters were exposed to Kodak XAR film, and colonies which did not bind phospho-CREB were identified. The corresponding cDNAs were sequenced on both strands.

Phosphatase protection assays. ^{32}P-labeled CREB (0.25 pmol) was mixed with 100 pmol of GST fusion proteins in a total volume of 60 μl of phosphatase buffer (20 mM HEPES [pH 7.0], 100 mM KCl, 0.1 mg of BSA per ml, 1 mM dithiothreitol, 7 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 5 ng of leupeptin per ml). After 30 min of incubation at 37°C, protein phosphatase 2A (PP2A) (0.2 μg, kindly provided by G. Walter) was added to each sample, and CREB dephosphorylation was allowed to proceed at 37°C. Time point samples were collected at 15-min intervals up to 2 h, and reactions were terminated by addition of SDS loading buffer. The samples were loaded onto SDS-polyacrylamide gels and exposed to X-ray film.

Microinjection assays. NIH 3T3 cells were cultured in 7.5% CO_2 in Dulbecco modified Eagle medium containing 10% calf serum. To facilitate microinjection, cells were plated on scored glass coverslips and made quiescent by serum deprivation. The cells were placed in Dulbecco modified Eagle medium containing 0.05% calf serum for 24 to 48 h prior to microinjection. The cells were microinjected with an Eppendorf semiautomated microinjection system that delivers $\sim 10^{-15}$ liters per cell. The pCRE-β-galactosidase reporter construct (200 ng/μl) (14) was injected along with a marker rabbit immunoglobulin G (IgG). The M4B and KIX peptides were injected at equal concentrations of 5 mg/ml. M4B is a mutant KIX peptide, identified by PCR mutagenesis screening, with the inactivating point mutations I-611→T and Y-650→N. After injection, cells were immediately stimulated with 10 μM forskolin and 0.5 mg of 3-isobutyl-1-methylxanthine (IBMX) per ml for 2 h to induce cAMP-dependent transcription. Subsequently, cells were fixed in 3.7% formaldehyde–PBS, permeabilized with 0.3% Triton X-100, and immunostained with donkey anti-rabbit IgG–fluorescein isothiocyanate (1:200) (Jackson Laboratories) to detect injected cells. Cells that contained a blue precipitate were scored positive. As a control, cells were microinjected with a constitutive expression construct, pCMX-β-galactosidase (100 ng/μl), and the KIX peptide at the same concentration as above (5 mg/ml). Two hours after injection, the cells were fixed and stained as described above.

RESULTS

An α-helical domain in CBP binds to phospho-Ser-133 CREB. To identify a minimal domain in CBP which is both necessary and sufficient for interaction with CREB phosphorylated at Ser-133 (phospho-Ser-133 CREB), we prepared a series of recombinant polypeptides (4) (Fig. 1A). In GST pull-down assays, a 94-aa fragment of CBP (KIX 10.4) extending from aa 586 to 679 of CBP was sufficient for high-affinity phospho-CREB binding activity. Further carboxy-terminal truncation of the KIX polypeptide to residue 661 (KIX 5.6 [aa 576 to 661]) lowered the binding affinity of KIX for phospho-Ser-133 CREB three- to fourfold (Fig. 1B). N-terminal deletion to aa 616 similarly reduced complex formation. Thus, 94 residues in KIX (aa 586 to 679) appear to be required for phospho-CREB recognition, a result which is further supported by sequence alignment data revealing that CBP, its paralog P300, and a related protein in *Caenorhabditis elegans* (R10e11.1) have strong sequence identity in that region (not shown). In parallel binding assays, we determined that the KIX-related region of P300 could indeed associate with phospho-Ser-133 CREB (not shown).

Previous studies demonstrating that the 72-aa KID in CREB is both necessary and sufficient for transcriptional induction by cAMP prompted us to test this domain for interaction with CBP (Fig. 1C). In solution binding assays, a recombinant KID polypeptide (aa 88 to 160), phosphorylated in vitro at Ser-133 with PKA, interacted strongly with CBP (Fig. 1C, lane 2). But the unphosphorylated KID did not bind to CBP (Fig. 1C, lane KID), demonstrating that formation of this complex is also phospho-Ser-133 dependent.

To examine the structural basis for interaction between CBP and CREB, we performed circular dichroism studies of the KIX and KID peptides (Fig. 1D). The KIX domain in CBP appeared to be α helical, as indicated by a maximum at 194 nm and two minima at 207 and 223 nm. By contrast, the KID in CREB showed no evidence of ordered structure, as revealed by a minimum at 203 nm. And PKA phosphorylation at Ser-133 had no effect on the circular dichroism spectrum of the KID, suggesting that phosphorylation does not induce structural changes in the KID. This result is supported by preliminary one-dimensional nuclear magnetic resonance studies of the KID in CREB which suggest that the KID is largely unstructured in both the unphosphorylated and phosphorylated states (14a).

Hydrophobic interactions contribute to CREB-CBP complex formation. To identify individual residues in the KIX polypeptide which are important for interaction with CREB, we developed a colony binding assay (Fig. 2). Using ^{32}P-labeled CREB as a probe, we detected specific binding to bacterial colonies which expressed recombinant KIX protein but not to colonies expressing unrelated proteins. By employing a random PCR mutagenesis protocol, we generated point mutations within the KIX domain (aa 455 to 679) and identified mutant KIX cDNAs which expressed the full-length KIX polypeptide when purified by glutathione-Sepharose chromatography but did not bind Ser-133 phospho-CREB in vitro (Fig. 2B). Sequence analysis of these phospho-CREB binding mutants revealed a cluster of amino acid substitutions occurring primarily at conserved hydrophobic residues from aa 591 to 620 of the KIX domain. On the basis of the helicity predicted for KIX by circular dichroism spectroscopy, most of these hydrophobic residues appear to occupy one face of an amphipathic α helix.

To determine whether hydrophobic interactions are important for complex formation between CBP and phospho-Ser-133 CREB, as predicted from the mutagenesis studies, we examined the stability of this complex in the presence of increasing concentrations of NaCl (Fig. 2D). Binding of phospho-CREB to a GST-KIX affinity resin was stable at high concentrations of salt (500 mM NaCl) (Fig. 2D) but was readily disrupted by a nonionic detergent (0.1% deoxycholate) (Fig. 2E). These results support the proposal that hydrophobic interactions contribute importantly to the formation of the CREB-CBP complex. In this regard, the KID in CREB (aa 88 to 160) also contains a number of hydrophobic residues which flank the Ser-133 phosphoacceptor site and which are conserved in the CREB family members CREM and ATF1. Whereas the wild-type phospho-Ser-133 KID could bind with high affinity to GST resins containing the KIX S/B polypeptide (Fig. 2F, left panel, lane KIX S/B), a mutant KID peptide containing Ile-137–Leu-138 to Ala-137–Ala-138 substitutions could not (Fig. 2F, right panel, lane KIX S/B). These results indicate that hydrophobic residues flanking phospho-Ser-133 are indeed important for complex formation.

The KIX polypeptide is recruited to cAMP-responsive promoters and blocks cAMP-responsive transcription in vivo. To determine whether we could visualize phosphorylation-dependent recruitment of KIX to a cAMP-responsive promoter, we performed gel shift assays using a double-stranded cAMP-responsive element (CRE) oligonucleotide (Fig. 3A and B). Addition of the KIX polypeptide to reaction mixtures containing phospho-Ser-133 CREB and a ^{32}P-labeled CRE generated

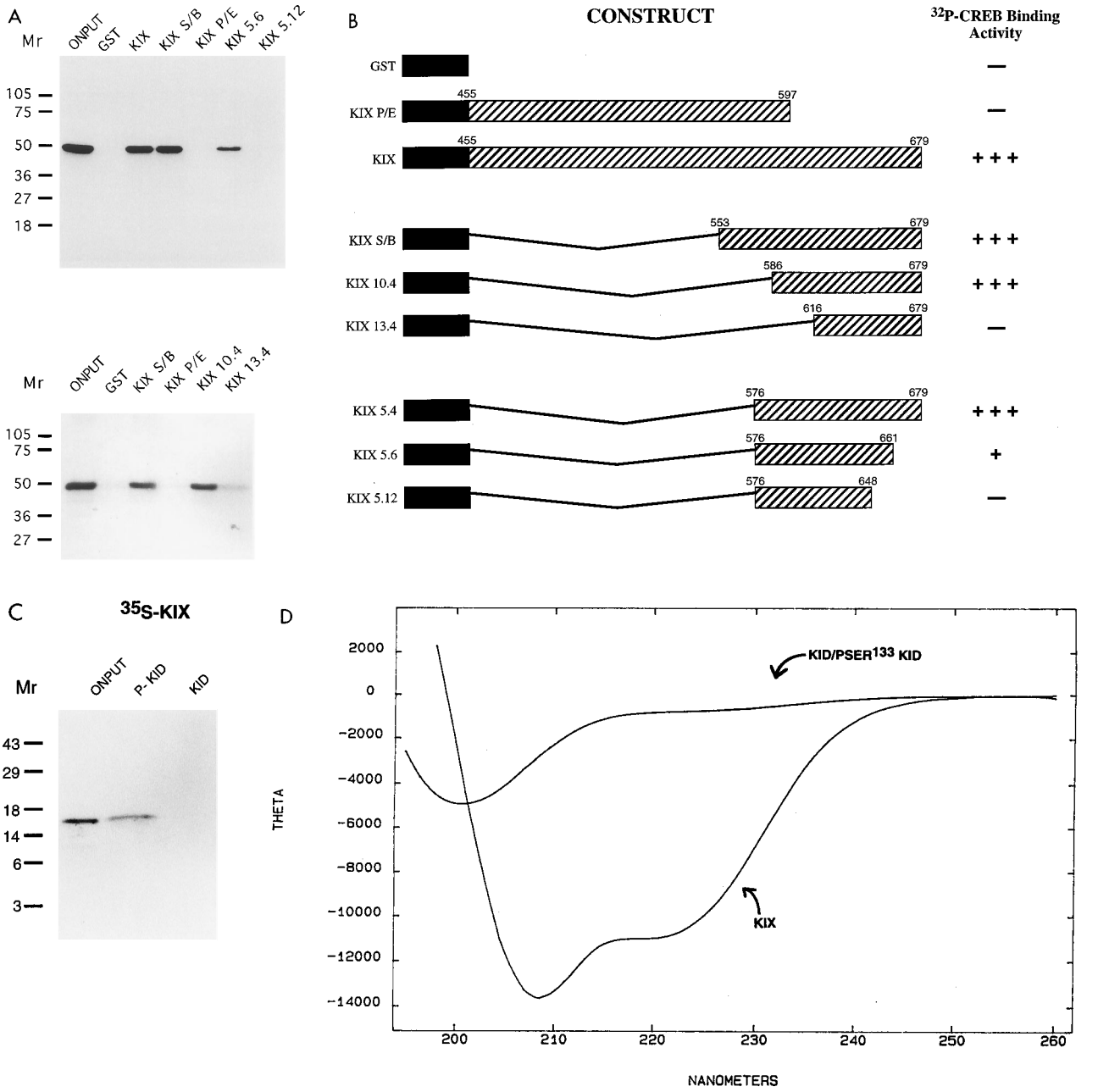


FIG. 1. The KIX region in CBP is an α-helical domain which binds to the KID of CREB in a phospho-Ser-133-dependent manner. (A and B) Characterization of domains in CREB and CBP which are necessary and sufficient for phosphorylation-dependent association in vitro. (A) GST pull-down assay of ^{32}P-labeled CREB phosphorylated at Ser-133 in vitro with PKA. Lanes: ONPUT, ^{32}P-labeled CREB added to each resin; GST, control resin containing GST alone; KIX, GST affinity resin expressing a CBP fragment consisting of aa 455 to 679; KIX S/B, GST affinity resin expressing a CBP fragment consisting of aa 553 to 679; KIX P/E, GST affinity resin expressing a CBP fragment consisting of aa 455 to 597; KIX 5.6, GST affinity resin expressing a CBP fragment consisting of aa 576 to 661; KIX 5.12, GST affinity resin expressing a CBP fragment consisting of aa 576 to 648; KIX 10.4, GST affinity resin expressing a CBP fragment consisting of aa 586 to 679; KIX 13.4, GST affinity resin expressing a CBP fragment consisting of aa 616 to 679. Mr, relative molecular weight in thousands. (B) Summary of deletional analysis of the KIX domain by GST pull-down assays. +++, 50 to 100% of wild-type binding activity; ++, 15 to 50% of wild-type binding activity; +, 5 to 15% of wild-type binding activity; −, no binding activity. Inclusive amino acid endpoints for each construct are indicated. (C) Pull-down assay of purified recombinant ^{35}S-labeled KIX S/B polypeptide. Lanes: ONPUT, ^{35}S-labeled KIX added to each resin; P-KID, retention of KIX on resin containing Ser-133-phosphorylated KID peptide (aa 88 to 160); KID, retention of KIX on resin containing unphosphorylated KID peptide. (D) Structure of the KID and the KIX domain in vitro. Circular dichroism spectra of purified KIX S/B (aa 553 to 679) and phospho-Ser-133 KID (aa 88 to 160) polypeptides are shown. The spectra are plotted as molar ellipticity (THETA) versus wavelength in nanometers. α-helical content was estimated by PROSEC analysis (3).

a complex which migrated more slowly than the phospho-CREB–CRE complex (Fig. 3A; compare lanes 1 and 5). But addition of the KIX polypeptide to reaction mixtures containing unphosphorylated CREB had no effect on the mobility of the CREB-CRE complex (Fig. 3A; compare lanes 1 and 6), suggesting that KIX is recruited to cAMP-responsive promoter elements in a phospho-Ser-133-dependent manner. Moreover, CREB binding mutants M4B and KIX P/E showed no supershifting activity when added to phospho-Ser-133 CREB–CRE complexes (Fig. 3B, lanes marked M4B and KIX P/E), indi-

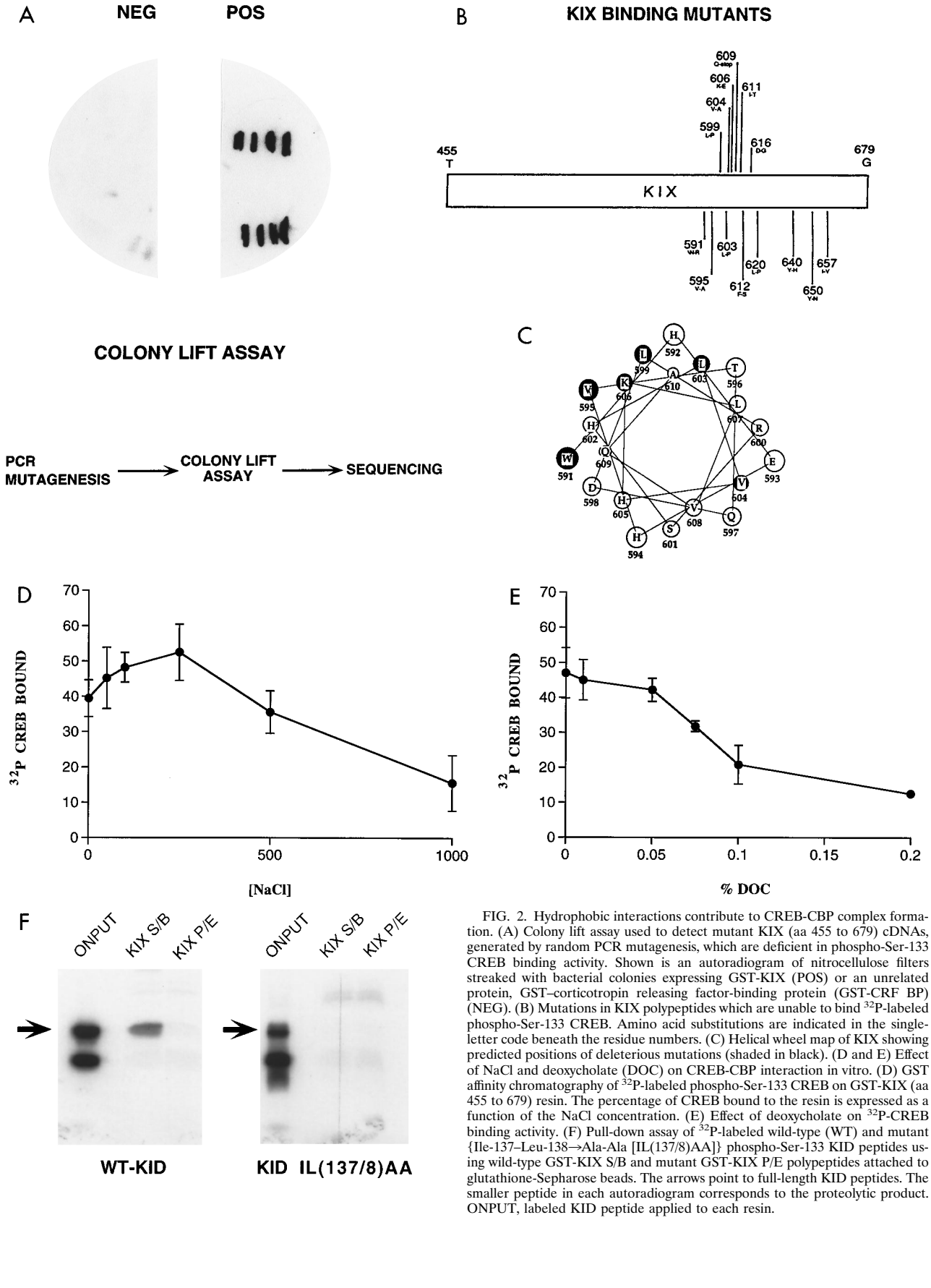


FIG. 2. Hydrophobic interactions contribute to CREB-CBP complex formation. (A) Colony lift assay used to detect mutant KIX (aa 455 to 679) cDNAs, generated by random PCR mutagenesis, which are deficient in phospho-Ser-133 CREB binding activity. Shown is an autoradiogram of nitrocellulose filters streaked with bacterial colonies expressing GST-KIX (POS) or an unrelated protein, GST–corticotropin releasing factor-binding protein (GST-CRF BP) (NEG). (B) Mutations in KIX polypeptides which are unable to bind ^{32}P-labeled phospho-Ser-133 CREB. Amino acid substitutions are indicated in the single-letter code beneath the residue numbers. (C) Helical wheel map of KIX showing predicted positions of deleterious mutations (shaded in black). (D and E) Effect of NaCl and deoxycholate (DOC) on CREB-CBP interaction in vitro. (D) GST affinity chromatography of ^{32}P-labeled phospho-Ser-133 CREB on GST-KIX (aa 455 to 679) resin. The percentage of CREB bound to the resin is expressed as a function of the NaCl concentration. (E) Effect of deoxycholate on ^{32}P-CREB binding activity. (F) Pull-down assay of ^{32}P-labeled wild-type (WT) and mutant {Ile-137–Leu-138→Ala-Ala [IL(137/8)AA]} phospho-Ser-133 KID peptides using wild-type GST-KIX S/B and mutant GST-KIX P/E polypeptides attached to glutathione-Sepharose beads. The arrows point to full-length KID peptides. The smaller peptide in each autoradiogram corresponds to the proteolytic product. ONPUT, labeled KID peptide applied to each resin.

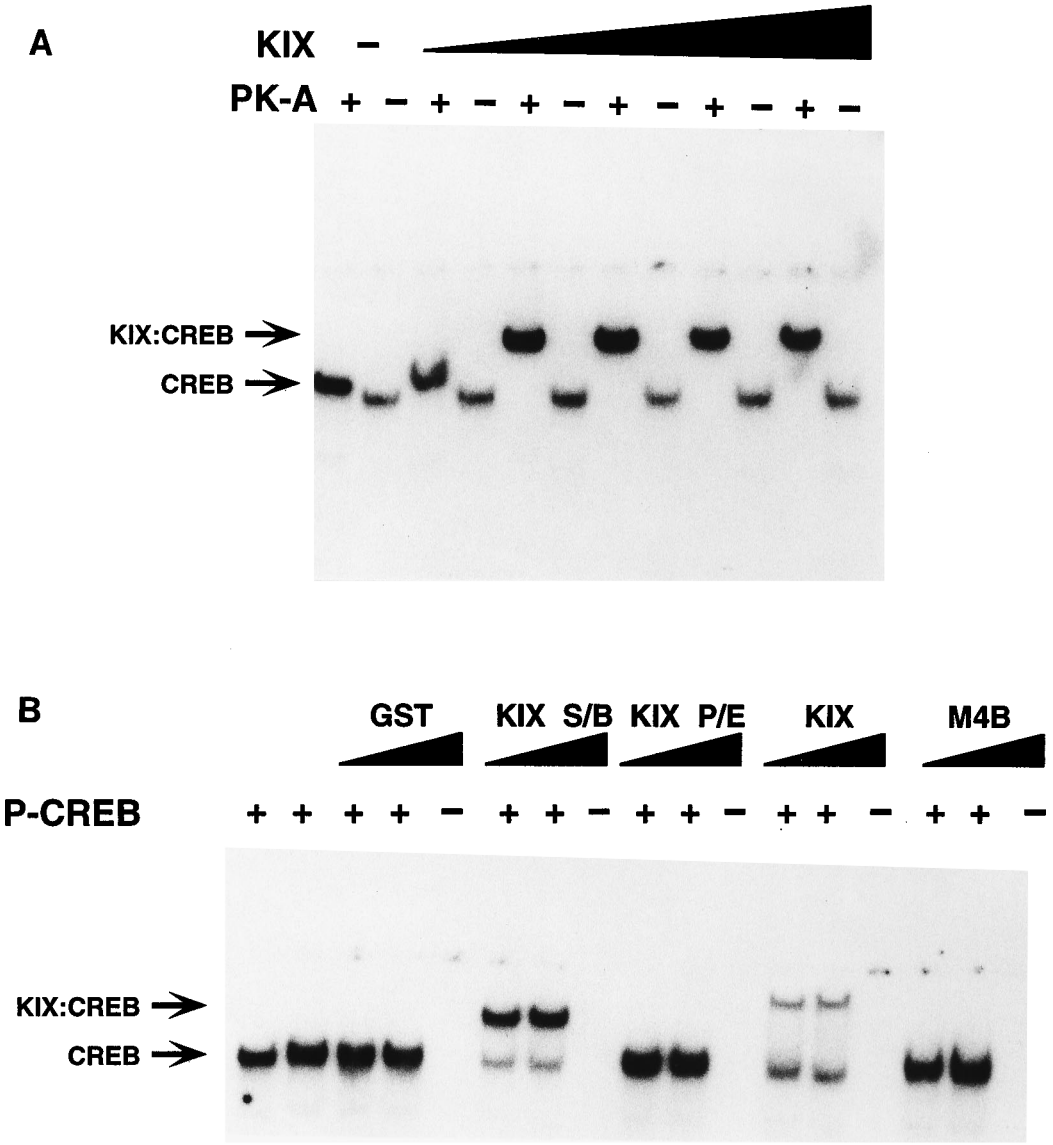


FIG. 3. The KIX polypeptide is recruited to cAMP-responsive promoters in a phospho-Ser-133 CREB-dependent manner and inhibits transcriptional induction by cAMP in vivo. (A and B) Gel shift assay with a somatostatin gene CRE double-stranded oligonucleotide and recombinant CREB protein. (A) Effect of increasing concentrations of KIX (indicated by crescendo bar). +, reaction mixture containing PKA catalytic subunit; −, reaction mixture lacking PKA catalytic subunit. CREB indicates the position of the CREB-CRE complex. KIX:CREB indicates the position of the KIX–phospho-Ser-133 CREB–CRE complex. (B) Gel shift assay of wild-type and mutant KIX polypeptides in the presence of phospho-CREB (P-CREB) (+) or no CREB (−). Crescendo bars indicate increasing amounts of protein in the gel shift assay. Arrows point to CREB-CRE (CREB) or KIX-CREB-CRE (KIX:CREB) complexes. GST, GST alone; KIX S/B, aa 553 to 679; KIX P/E, aa 455 to 679; KIX, aa 455 to 679; M4B, mutant KIX polypeptide (identified in PCR mutagenesis screen) which contains two deleterious point mutations (I-611→T and Y-650→N). (C to E) Microinjection of the KIX peptide inhibits cAMP-inducible transcription. Quiescent NIH 3T3 fibroblast nuclei were microinjected with the pCRE-β-galactosidase reporter plasmid, marker IgG, and either a mutant peptide, M4B (C), or the KIX peptide (D). The cells were stimulated with forskolin-IBMX for 2 h, fixed, and then assayed for β-galactosidase activity. Subsequently, the cells were permeabilized and immunostained with a secondary anti-rabbit antibody conjugated to fluorescein isothiocyanate to determine the number of cells that had been microinjected. The dark cells in panel C were all injected with the M4B peptide; the cells in panel D that were injected with KIX are indicated by arrows. (E) As a control, constitutive reporter activity was assessed in the presence of the KIX peptide. Cells were microinjected with pCMX-β-galactosidase, marker IgG, and the KIX peptide. All of the dark cells in panel E were microinjected, and no inhibition by the peptide was observed.

cating that the recruitment of KIX to such complexes occurs via a direct interaction with phospho-CREB.

The ability of KIX to be recruited to a cAMP-responsive promoter element prompted us to test whether KIX can inhibit cAMP-responsive transcription by competing for interaction between cellular CBP and CREB (Fig. 3C to E). NIH 3T3 fibroblasts were microinjected with the forskolin- and IBMX-inducible pCRE-β-galactosidase reporter construct along with either the KIX S/B polypeptide or the CREB binding mutant M4B polypeptide, which contains two inactivating point mutations (I-611→T and Y-650→N). Microinjection of the KIX S/B peptide inhibited reporter activity in response to the agonist (Fig. 3D), whereas the mutant KIX M4B peptide had no effect on pCRE-β-galactosidase activity (Fig. 3C). Of the 147 cells injected with the M4B peptide, 92.3% ± 3.9% (mean ± standard deviation) displayed cAMP-dependent transcription, as

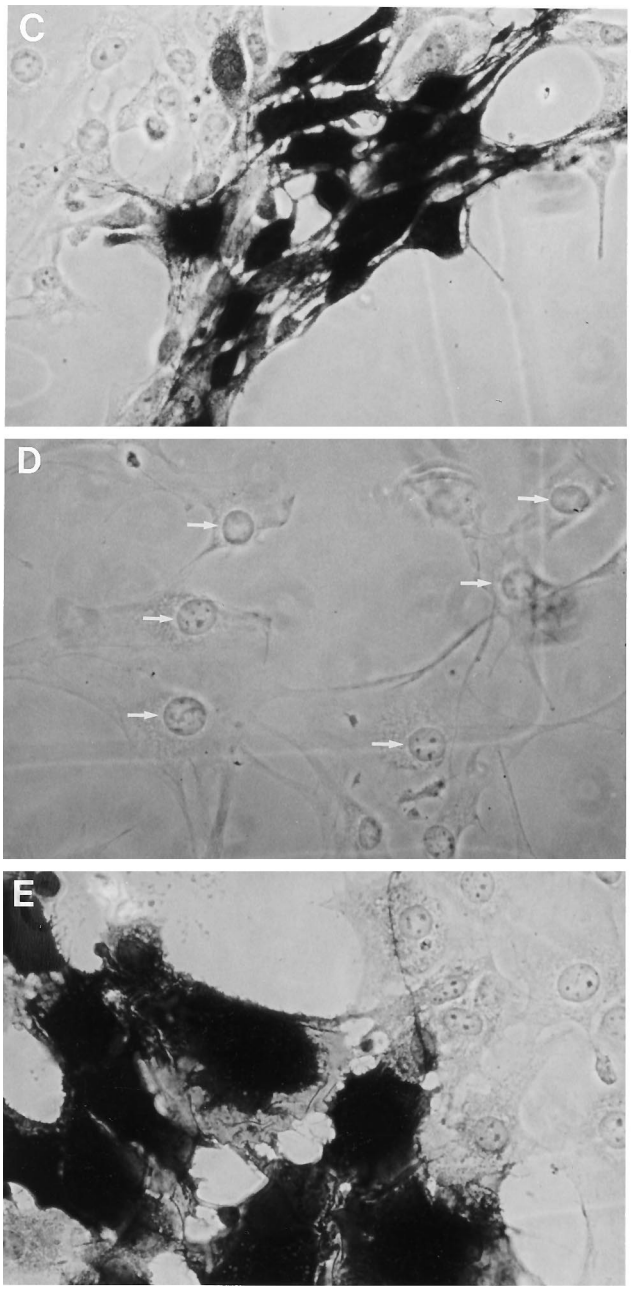


FIG. 3—*Continued.*

determined by the number of blue cells, compared with only 0.5% ± 0.3% of the 415 cells that were microinjected with the KIX peptide. Microinjection of the KIX peptide had no effect on β-galactosidase activity derived from a constitutive cytomegalovirus promoter construct (Fig. 3E). In that case, 100% ± 0% of the 159 cells injected were blue. These data suggest that the ability of KIX to block cAMP-responsive transcription in vivo is consistent with its ability to associate with phospho-CREB in vitro.

The PKA phosphoacceptor site in CREB participates directly in binding to CBP. The relatively large region of CREB involved in binding to CBP (KID [aa 88 to 160]) prompted us to examine whether smaller CREB peptides might also bind to CBP in a phosphorylation-dependent manner. In this experiment, small phospho-Ser-133 CREB peptides could indeed bind to KIX, albeit with a lower affinity than full-length CREB. The dissociation constant (K_d) of phospho-Ser-133 $CREB_{121–151}$ for binding to KIX, for example, was 6.5 μM, compared with 32 nM for full-length phospho-Ser-133 CREB (Fig. 4). A 14-aa phospho-Ser-133 $CREB_{128–141}$ peptide recognized KIX with a K_d of 0.56 mM, suggesting that sequences flanking the Ser-133 phosphoacceptor site stabilized complex formation. However, none of the unphosphorylated CREB peptides showed detectable binding to KIX (not shown), demonstrating that complex formation was phosphorylation dependent.

The ability of a 4-aa $CREB_{131–134}$ peptide to recognize KIX in a phospho-Ser-133-dependent manner (Fig. 4B), albeit with a low affinity, indicated that phospho-Ser-133 may participate directly in binding of CREB to CBP. However, free phosphoserine was unable to compete for binding of phospho-CREB to KIX in displacement assays (not shown), suggesting that the interaction of phosphoserine with KIX may be either weak or too unstable to detect by this method. To further test this model, we employed a phosphatase protection assay using the Ser/Thr protein phosphatases PP1 and PP2A, which have been shown to dephosphorylate CREB at Ser-133 in vitro and in vivo (Fig. 5). When added to ^{32}P-labeled phospho-Ser-133 CREB, both phosphatases stimulated Ser-133 dephosphoryla-

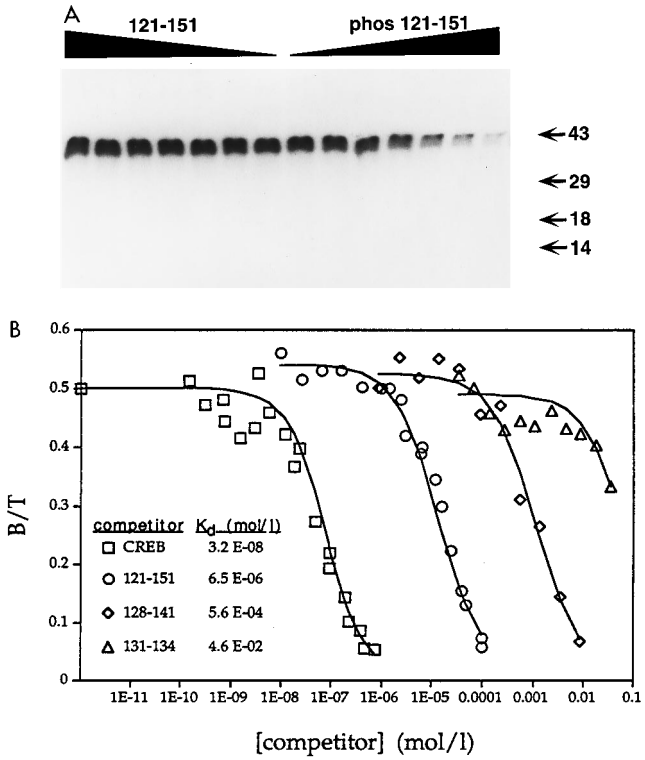


FIG. 4. Flanking residues in CREB stabilize phosphorylation-dependent interaction with KIX. (A) Typical autoradiogram showing results of a displacement assay with phosphorylated (phos 121-151) or unphosphorylated (121-151) $CREB_{121–151}$ peptide. The effect of increasing amounts of CREB peptide (indicated by crescendo bars) on binding of ^{32}P-labeled phospho-Ser-133 CREB to KIX is shown. Molecular weights in thousands are indicated on the right. (B) Binding of ^{32}P-phosphorylated CREB to GST-KIX S/B was measured in the presence of increasing concentrations of unlabeled phospho-CREB (CREB) or phospho-Ser-133 peptides representing the indicated amino acids of the KID. The displacement curves were analyzed by the LIGAND program, and the resulting best-fit curves are shown. The calculated K_ds for the various competitors are also given. B/T, bound/total (fraction of ^{32}P-labeled CREB bound to KIX resin).

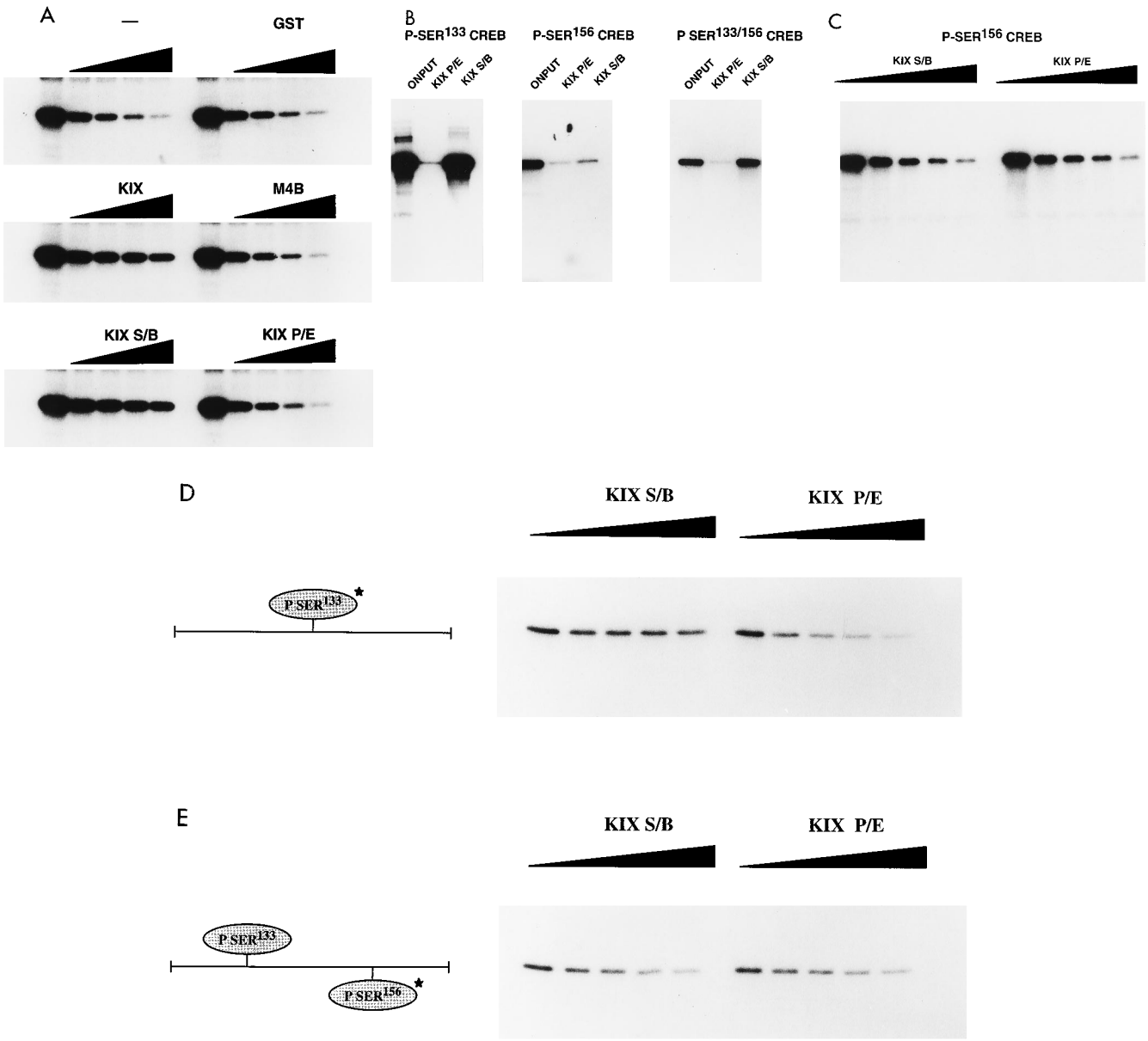


FIG. 5. The KIX domain forms unique contacts with the PKA phosphoacceptor Ser-133 in CREB. (A) Phosphatase protection assay with recombinant ^{32}P-labeled CREB phosphorylated in vitro with PKA. Phospho-CREB was coincubated with the Ser/Thr protein phosphatase PP2A for increasing times (as indicated by crescendo bars) in the presence of various polypeptides indicated above the crescendo bars. Samples were then analyzed by SDS-PAGE. −, no added protein; GST, control; KIX, purified KIX polypeptide (aa 455 to 679); M4B, KIX mutant identified in PCR mutagenesis screen and containing two inactivating point mutations (I-611→T and Y-650→N); KIX S/B, aa 553 to 679; KIX P/E, aa 455 to 597. (B and C) The casein kinase II phosphoacceptor site in CREB (Ser-156) does not associate with CBP and is not protected from dephosphorylation by PP2A. (B) GST affinity chromatography of PKA-phosphorylated (P-SER^{133} CREB), casein kinase II-phosphorylated (P-SER^{156} CREB), or doubly phosphorylated (P SER$^{133/156}$ CREB) CREB. Lanes: ONPUT, total ^{32}P-labeled CREB applied to KIX resins; KIX P/E and KIX S/B, CREB binding to GST-KIX P/E and GST-KIX S/B resins, respectively. The autoradiogram shows ^{32}P-labeled CREB bound to each resin. (C) PP2A protection assay of Ser-156 phospho-CREB in the presence of the KIX S/B or KIX P/E polypeptide. Crescendo bars indicate increasing times of incubation (from 0 to 2 h) with PP2A. (D and E) Phospho-Ser-133 forms contacts with the KIX domain different from those formed by phospho-Ser-156. (D) PP2A-mediated dephosphorylation of CREB at Ser-133 in the presence of the wild-type KIX S/B or mutant KIX P/E polypeptide. (E) PP2A-mediated dephosphorylation at Ser-156 of doubly phosphorylated (at Ser-133 and Ser-156) CREB as indicated in the diagram on the left. CREB was phosphorylated at Ser-156 with casein kinase II. In panels D and E, asterisks denote sites phosphorylated with ^{32}P-labeled ATP, and crescendo bars indicate increasing incubation times from left to right.

tion in a linear fashion over a 2-h period (Fig. 5A, panel −). When these phosphatases were coincubated with the KIX or KIX S/B polypeptide, however, CREB dephosphorylation by the phosphatases was markedly inhibited (Fig. 5A, panels KIX and KIX S/B). By contrast, mutant KIX polypeptides M4B and P/E, which do not bind phospho-Ser-133 CREB, had no effect on the rate of CREB dephosphorylation (Fig. 5A, panels M4B and KIX P/E), suggesting that KIX may form unique contacts with phospho-Ser-133, perhaps in the form of a pocket.

To test whether other residues in the KID interact similarly with KIX, we prepared ^{32}P-labeled CREB phosphorylated at Ser-156 by casein kinase II. Phospho-Ser-156 CREB did not bind to GST-KIX in pull-down assays (Fig. 5B; compare lanes 3 in panels P-SER^{133} CREB and P-SER^{156} CREB), and cor-

respondingly, the KIX polypeptide could not protect CREB from PP2A-mediated dephosphorylation at Ser-156 (Fig. 5C). Upon phosphorylation with PKA using unlabeled ATP, the doubly phosphorylated phospho-Ser-133/phospho-Ser-156 CREB protein showed a wild-type level of binding to GST-KIX resins (Fig. 5B, panel P $SER^{133/156}$ CREB, lane 3). But KIX did not protect the doubly phosphorylated CREB from dephosphorylation at Ser-156 (Fig. 5D and E), demonstrating that phospho-Ser-133 forms specific contacts with residues in KIX.

To further investigate whether phospho-Ser-133 directly participates in binding to CBP, we performed cross-linking assays using platinum tetrachloride, a reagent with high affinity for sulfhydryl groups (5) (Fig. 6A). The absence of sulfur-containing residues in the $CREB_{121–151}$ peptide prompted us to construct a platinum adduct with this peptide following its thiophosphorylation at Ser-133 with γ-^{35}S-ATP. In solution binding assays, phosphorylated and thiophosphorylated $CREB_{121–151}$ peptides bound with comparable affinities to KIX (not shown). When incubated with the purified KIX peptide (14.3 kDa) in the presence of platinum tetrachloride, the ^{35}S-labeled 3.6-kDa $CREB_{121–151}$ thiophosphorylated peptide gave rise to an 18-kDa cross-linked product (Fig. 6A, lane 4) whose size is consistent with the predicted molecular mass for the thiophospho-Ser-133 $KID_{121–151}$–KIX complex. No cross-linked complex was detected in samples in which the ^{35}S-labeled CREB peptide was incubated with nonspecific proteins such as BSA or with the phospho-CREB-binding mutant peptide KIX P/E (not shown). To determine whether platinum-dependent cross-linking of $CREB_{121–151}$ to KIX occurred via the thiophosphate moiety at Ser-133 or via other residues in the peptide, we prepared ^{32}P-labeled phospho-Ser-133 $CREB_{121–151}$, which contained only a phosphate moiety at Ser-133. When incubated with the KIX polypeptide in the presence of platinum, phospho-Ser-133 $CREB_{121–151}$ did not form any covalent complexes (Fig. 6A, lane 6), demonstrating that thiophosphorylation at Ser-133 is essential for platinum-dependent cross-linking. The short bond distance for platinum (2 Å [0.2 nm]) compared with those of other cross-linking reagents indicates that phospho-Ser-133 is positioned next to residues in KIX which are important for complex formation.

To identify residues in KIX which interacted with the thiophosphorylated Ser-133, we subjected the platinum-cross-linked $CREB_{121–151}$–KIX complex to trypsin proteolysis. Following their resolution by reverse-phase high-performance liquid chromatography (HPLC), individual tryptic peptides were evaluated for the presence of cross-linked ^{35}S-labeled phospho-Ser-133 by scintillation counting (Fig. 6B). We identified one ^{35}S-labeled peptide which contained two peptide sequences: SHLVXK, corresponding to aa 601 to 606 (SHLVHK) in CBP, and XPXY, corresponding to aa 131 to 134 (RPSY) in CREB. In the process of examining sequences surrounding the KIX tryptic peptide (aa 601 to 606), we noticed an adjacent arginine residue (Arg-600) which was conserved in P300 and in the *C. elegans* homolog R10e11.1. The unique capacity of arginine residues to form ion pairs with phosphorylated amino acids prompted us to examine whether Arg-600 was important for phospho-CREB recognition. Mutagenesis of Arg-600 to Gln severely disrupted complex formation with phospho-Ser-133 CREB in far Western blotting and solution binding assays (Fig. 6C, fractions 7 to 9). These results indicate that Arg-600 is indeed critical for phosphoserine recognition.

DISCUSSION

Serine phosphorylation has been shown to regulate protein activity by at least two different mechanisms: direct and allosteric. For example, phosphorylation of glycogen phosphorylase at Ser-14 augments catalytic activity via an allosteric mechanism which involves altered subunit interactions (16). By contrast, phosphorylation of isocitrate dehydrogenase at Ser-113 causes few changes in its tertiary structure (10). Ser-113 is located within the active site of isocitrate dehydrogenase, and phosphorylation there inhibits enzyme-substrate interactions by electrostatic repulsion.

Previous experiments with CREB had prompted us to speculate that phosphorylation might regulate this protein via an allosteric mechanism. This model was supported by mutagenesis data showing that acidic residues could not substitute for Ser-133 (7). Moreover, the presence of a constitutive activation domain in CREB termed Q2 (aa 161 to 283), which is removed from the PKA site, suggested that phosphorylation might regulate CREB by exposing this domain to the transcriptional apparatus. More recent experiments have revealed that the KID and Q2 can synergize not only in *cis* but also in *trans* in response to PKA induction (2), providing strong evidence against this allosteric model. In this study, circular dichroism and one-dimensional nuclear magnetic resonance analyses revealed that the KID assumes a disordered structure which is unaffected by Ser-133 phosphorylation.

Ser-133 phosphorylation appears to promote interaction with CBP via a direct mechanism. In this regard, phosphatase protection assays revealed that KIX interacts uniquely with phospho-Ser-133 upon binding to the KID. Indeed, a small 4-aa CREB peptide (aa 131 to 134) interacts with CREB in a phosphorylation-dependent manner, suggesting that phospho-Ser-133 drives the specificity of the interaction with CBP. This hypothesis was substantiated by platinum cross-linking experiments in which phospho-Ser-133 itself was found to come into close contact with residues 601 to 606 in the KIX polypeptide. Although we could not identify the specific amino acid which coordinates with platinum in these cross-linking experiments, the high affinity of platinum for imidazole rings implicates histidines 602 and 606 in this process. Indeed, the presence of a conserved arginine (Arg-600) which lies adjacent to the cross-linked tryptic KIX peptide and which is required for complex formation suggests that this residue may coordinate with phosphoserine 133 in CREB.

KIX has a number of interesting similarities to the phosphotyrosine binding src homology 2 (SH2) domain (15). The minimal KIX domain (KIX 10.4 [aa 586 to 679]) is similar in size to the SH2 motif (100 aa), and, like SH2 domains, it appears to recognize an unstructured region of CREB by binding directly to the phosphorylated amino acid (Ser-133). As with SH2 domains, high-affinity interaction between KIX and phospho-CREB requires residues which flank the Ser-133 phosphoacceptor site. These flanking residues may stabilize complex formation primarily via hydrophobic interactions. Ile-137 and Leu-138 in CREB, for example, were found to contribute importantly to high-affinity interaction with the KIX domain. Moreover, complex formation between KIX and phospho-Ser-133 CREB was found to be stable in the presence of high concentrations of KCl but susceptible to low levels of nonionic detergent.

How, then, does CBP recognize phospho-Ser-133 CREB? Structural studies reveal that, in contrast to the SH2 motif, KIX is highly helical. In particular, one segment of KIX (aa 590 to 610) may adopt an amphipathic helical form which is critical for complex formation. Indeed, the majority of phos-

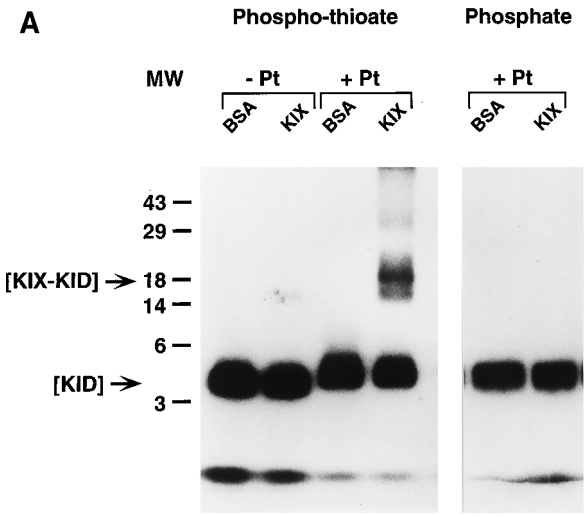
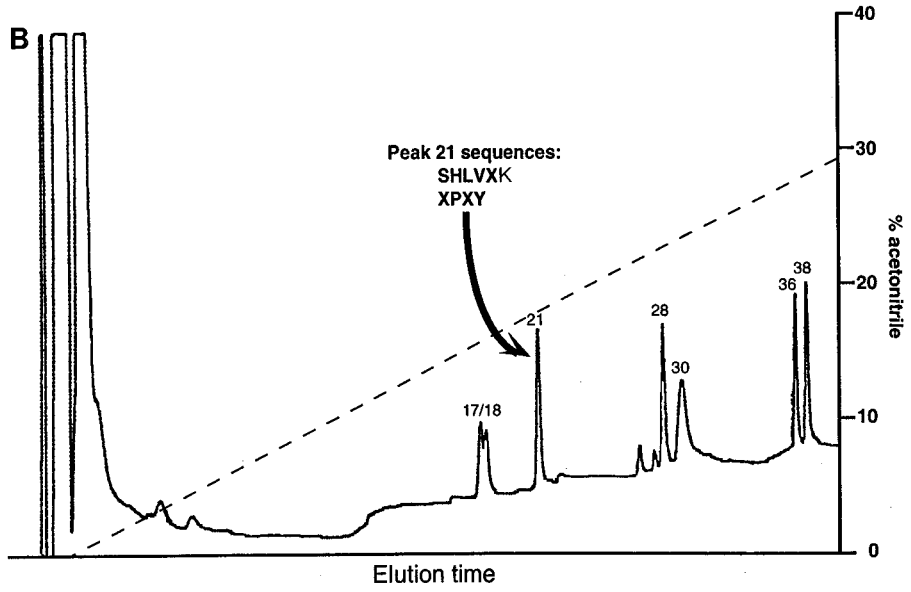
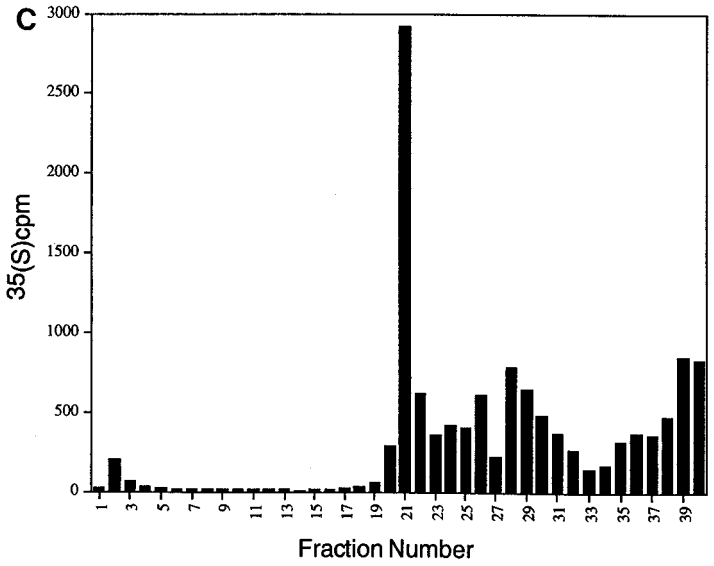
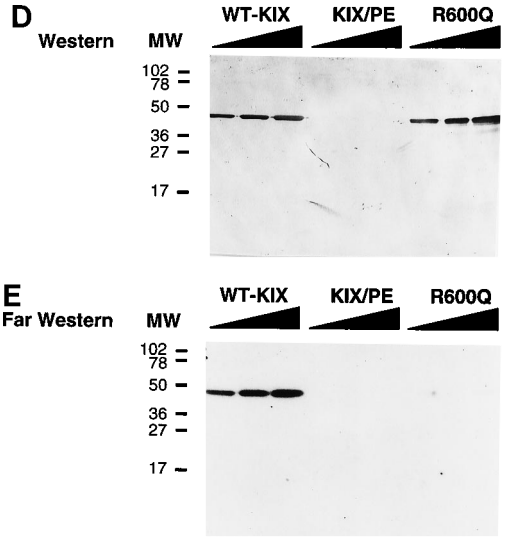


FIG. 6. Phospho-Ser-133 in CREB interacts directly with residues in CBP. (A) Cross-linking assay with $CREB_{121–151}$ and KIX S/B peptides. Phospho-thioate, thiophosphorylated $CREB_{121–151}$ labeled at Ser-133 with PKA by using γ^{35}S-ATP; Phosphate, phosphorylated $CREB_{121–151}$ labeled at Ser-133 with PKA by using [γ-^{32}P]ATP. The absence (− Pt) or presence (+ Pt) of platinum tetrachloride in binding reactions is indicated. MW, relative molecular weight in thousands. The phospho-$CREB_{121–151}$ peptide was incubated with BSA as a control or KIX peptide as indicated above each lane. The arrows point to the labeled phospho-$CREB_{121–151}$ peptide (KID) and the cross-linked KID-KIX complex (KIX-KID). (B) Reverse-phase HPLC analysis of peptides generated after trypsinization of the platinum-cross-linked KIX–^{35}S-phospho-KID complex. The dashed line indicates the ascending gradient of acetonitrile. The primary sequences derived after automated Edman degradation of peptides in peak 21 are indicated. SHLVXL corresponds to aa 601 to 606 of CBP, and XPXY corresponds to aa 131 to 134 of CREB. (C) Analysis of peptide fractions obtained from HPLC (B). The ^{35}S counts per minute correspond to ^{35}S-labeled thiophospho-Ser-133. (D and E) Arg-600 in KIX is critical for complex formation with phospho-Ser-133 CREB. (D) Western blot (immunoblot) assay of GST-KIX (WT-KIX), GST-KIX P/E, and GST-R600Q proteins with anti-CBP antiserum raised against the KIX S/B polypeptide. KIX P/E does not react with anti-CBP antiserum. (E) Far Western blot assay of wild-type and mutant KIX proteins with ^{32}P-labeled phospho-Ser-133 CREB as a probe. In panels D and E, crescendo bars indicate increasing amounts of purified recombinant proteins. MW, relative molecular weight in thousands.







pho-Ser-133 CREB binding mutants obtained from a random mutagenesis screen of KIX were targeted to hydrophobic residues within this amphipathic helix. These amino acids appear to cluster on a helical face opposite Arg-600, which we propose here to coordinate with phospho-Ser-133 in CREB. Thus, hydrophobic residues in the amphipathic helix (aa 590 to 610) may be more important for proper folding of the KIX domain than for direct interaction with hydrophobic residues in CREB.

The functional importance of CBP in cAMP-responsive transcription has been previously demonstrated by microinjection experiments in which anti-CBP antiserum abrogated the response to an agonist (1) and by transient-transfection assays in which overexpression of CBP potentiated CREB activity in a Ser-133-dependent manner (11). Our results reveal that CBP is recruited to cAMP-responsive elements upon phosphorylation of CREB. Furthermore, microinjection experiments with the KIX peptide demonstrate that this recruitment is critical for target gene induction in response to cAMP. Other growth factor pathways, such as those induced by the transforming growth factor β family, stimulate target cell responses via receptor Ser/Thr kinases (13). The intracellular events which follow transforming growth factor β induction remain to be elucidated, but it is tempting to speculate that, like the tyrosine kinase-linked receptors, these Ser/Thr kinase receptors may also induce protein-protein interactions via motifs which resemble the KIX domain.

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The first two authors contributed equally to this work.

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