Stimulus-specific Interaction between Activator-Coactivator Cognates Revealed with a Novel Complex-specific Antiserum*

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A number of second messenger pathways propagate inductive signals via protein-protein interactions that are phosphorylation-dependent. The second messenger, cAMP, for example, promotes cellular gene expression via the protein kinase A-mediated phosphorylation of cAMP-response element-binding protein (CREB) at Ser133, and this modification in turn stimulates the association of CREB with the co-activator, CREB-binding protein (CBP). The solution structure of the CREB-CBP complex, using relevant interaction domains, kinase inducible domain and kinase-induced domain interacting domain, referred to as KID and KIX, respectively, shows that KID undergoes a coil to helix transition, upon binding to KIX, that stabilizes complex formation. Whether such changes occur in the context of the full-length CREB and CBP proteins, however, is unclear. Here we characterize a novel antiserum that specifically binds to the CREB-CBP complex but to neither protein individually. Epitope mapping experiments demonstrate that the CREB-CBP antiserum detects residues in KID that undergo a conformational change upon binding to KIX. The ability of this antiserum to recognize full-length CREB-CBP complexes in a phoso-(Ser133)-dependent manner demonstrates that the structural transition observed with the isolated KID domain also occurs in the context of the full-length CREB protein. To our knowledge, this is the first report documenting formation of endogenous cellular protein-protein complexes in situ.

Although phosphorylation appears to enhance the nuclear import, multimerization, or DNA binding activities of certain factors, CREB belongs to a group of activators whose trans-activation potential is specifically affected (2).

The CREB trans-activation domain is bipartite, consisting of constitutive and inducible activators that function synergistically in response to cAMP stimulation (2, 3). The constitutive glutamine-rich activation domain, referred to as Q2, has been found to promote transcription via an interaction with TFIID (4). By contrast, the kinase inducible domain (KID) stimulates target gene expression, following its phosphorylation at Ser133, by associating with the KIX domain of the co-activator CBP (5–7). The solution structure of the KID-KIX complex reveals that Ser133 phosphorylated KID undergoes a random coil to helix transition upon complex formation with KIX, and this transition in turn stabilizes the interaction between CREB and CBP and its paralog P300 (8, 9).

In addition to cAMP, a wide variety of extracellular stimuli, including phosphoinositol and calcium agonists as well as certain growth factors such as nerve growth factor, epidermal growth factor, insulin-like growth factor, and platelet-derived growth factor, appear to promote Ser133 phosphorylation of CREB with high stoichiometry (10–14). However, these pathways are unable to promote target gene expression via CREB per se, reflecting a block either in recruitment of CBP or in the subsequent assembly of the transcriptional apparatus (10).

A number of methodologies, including co-immunoprecipitation (co-IP) and fluorescence resonance energy transfer (FRET), have been employed to evaluate protein-protein interactions (15). Such procedures are limited by technical manipulations, such as protein extraction (co-IP) or over-expression (FRET), that may influence the recovery or detection of protein complexes. Here we employ a novel complex-specific antiserum to monitor the CREB-CBP interaction following exposure to various stimuli. Immunocytochemical experiments reveal that CREB-CBP complex formation, in response to cAMP, is limited to discrete compartments within the nucleus. Remarkably, other stimuli were found to have distinct effects on complex formation, even in light of comparable potency at the level of Ser133 phosphorylation. Our results illustrate a novel mechanism for signal discrimination in the nucleus at the level of co-activator recruitment.

EXPERIMENTAL PROCEDURES

Preparation of CREB-CBP-specific Antiserum—KID and KIX were expressed in Escherichia coli BL21 cells and purified as described previously (8). The peptides were cross-linked with glutaraldehyde in cross-linking buffer (20 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl2, 2 mM EDTA). For anti-KID-KIX (aKK) antiserum, IgG was purified by 50% ammonium sulfate precipitation followed by protein A-agarose affinity purification. Antibodies to phoshoKID or KIX alone were preabsorbed by incubation with a phosphoKID and KIX-coupled Affi-Gel 10 resin individually. KID-KIX complex-specific antibodies were then purified by incubating with a phosphoKID-KIX-coupled Affi-Gel 10 resin and eluting with 100 mM glycine. For CREB/p300 co-immunoprecipitations, 100 ng of recombinant p300 (gift from P. Nakatani, Dana Farber Cancer Center) and 2 μg of recombinant CREB or phospho-(Ser133) CREB were co-incubated, and the immunoprecipitates were processed as described previously (16). Gel shift assays with2P-labeled CRE or rum albumin; CREM, mammalian CREB homolog; eCREB, Caenorhabditis elegans CREB polypeptide.

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Formation of CREB-CBP Complexes

**RESULTS**

To evaluate CREB-CBP complex formation in vivo, we developed a complex-specific antiserum using glutaraldehyde cross-linked phospho-(Ser\textsuperscript{133}) KID-KIX complexes as immunogen. αKK antiserum was initially purified from crude serum of immunized rabbits by chromatography over separate KID and KIX resins to remove antibodies that could recognize either phospho-(Ser\textsuperscript{133}) KID or KIX peptides independently (Fig. 1A). Flow-through fractions from these columns were then passed over resin containing cross-linked KID-KIX peptides, and the bound antibody fraction, referred to as αKK antiserum, was acid eluted. In Western blot assays, αKK antiserum could recognize cross-linked KID-KIX complexes but not KIX or phospho-(Ser\textsuperscript{133}) KID peptides alone (Fig. 1B). Consistent with the notion that αKK antiserum is also competent to detect complex formation between full-length phospho-(Ser\textsuperscript{133}) CREB and CBP/P300 proteins, P300 was recovered from immunoprecipitates of recombinant P300 and phospho-(Ser\textsuperscript{133}) CREB but not of P300 plus phospho-(Ser\textsuperscript{133}) CREB (Fig. 1C, top panel). αKK antiserum also detected the phospho-(Ser\textsuperscript{133})-dependent re-cruitment of CBP in immunoprecipitation assays, demonstrating the capacity of this antiserum to recognize complexes formed with both co-activators (Fig. 1C, bottom panel).

The KID domain is highly conserved among CREB family members, particularly in residues that function in protein interactions with KIX (8). In gel mobility shift assays, for example, αKK antiserum was also capable of binding to KIX complexes formed with the mammalian CREB homolog, CREM, but not with the more distantly related Caenorhabditis elegans CREB polypeptide (eCREB)\textsuperscript{2} (Fig. 2A). Compared with its mammalian counterpart, eCREB contains a number of amino acid substitutions within its KID domain (amino acids 40–65) (Fig. 2B), prompting us to evaluate which of these constituted an important epitope for αKK recognition.

Mutation of residues 121–123 (M1) in the a\textalpha region of rat CREB (amino acids 121–129) to corresponding amino acids of C. elegans CREB had no effect on either complex formation with KIX or recognition by αKK by gel shift assay (Fig. 2, B and C; compare WT and M1). Mutation of residues 127 and 129 (M2) or 136 and 139 (M3) in KID partially disrupted interaction with KIX by gel shift assay (Fig. 2, B and C; compare WT, M2, and M3). Although these residues do not appear to form surface contacts with KIX (8), mutation at these amino acids may impose structural constraints on the mutant KID peptides that make complex formation less favorable. Nevertheless, complexes formed with mutant M2 KID were supershifted by αKK antiserum, but complexes formed with mutant M3 KID containing Lys\textsuperscript{136} → Met and Asn\textsuperscript{139} → Lys substitutions in the a\textbeta region were not (Fig. 2C; compare WT, M2, and M3). Taken together, these results indicate that residues Lys\textsuperscript{136} and Asn\textsuperscript{139} are critical for recognition by αKK antiserum.

Lys\textsuperscript{136} and Asn\textsuperscript{139} are directly aligned on the solvent face of helix aβ, a region in KID that undergoes a random coil to helix transition upon complex formation with KIX (Fig. 2D). The importance of these residues for recognition by αKK suggests that the antiserum detects, in part, the conformational change in KID that accompanies complex formation with KIX. Moreover, the ability of αKK to recognize full-length CREB-CBP complexes suggests that the structural change detected by NMR analysis with KID and KIX peptides also occurs in the context of the full-length proteins.

In addition to cAMP, other stimuli such as the phorbol ester TPA can promote Ser\textsuperscript{133} phosphorylation of CREB, yet these stimuli are unable to induce target gene activation via CBP, reflecting a block either in CREB-CBP complex formation or in the subsequent recruitment of the transcriptional apparatus. To evaluate formation of CREB-CBP complexes in vivo, we employed NIH 3T3 cells expressing chromosomal copies of the rat somatostatin gene, hereafter referred to as D5 cells (17). Treating D5 cells with TPA induced Ser\textsuperscript{133} phosphorylation of CREB with comparable stoichiometry to forskolin by Western blot assay with phospho-specific CREB antiserum 5322 (Fig. 3A). Forskolin stimulated somatostatin mRNA accumulation 5-fold in D5 cells, whereas TPA had no discernible effect (Fig. 3B).

Consistent with the absence of phospho-(Ser\textsuperscript{133}) CREB staining under basal conditions, no CREB-CBP complexes were detected in untreated D5 cells by immunofluorescence analysis with αKK antiserum (Fig. 4). Treatment with forskolin induced accumulation of phospho-(Ser\textsuperscript{133}) CREB and correspondingly promoted the appearance of CREB-CBP complexes (Fig. 4A). By contrast with forskolin, however, no CREB-CBP complexes were detected in TPA-treated cells despite comparable levels of Ser\textsuperscript{133} phosphorylation.

To confirm the specificity of the αKK antiserum, we employed fibroblasts from CREB−/− mice (18). Compared with cells from wild-type littermates, which showed abundant nuclear staining following treatment with cAMP agonist, only background cytoplasmic staining was observed in CREB−/−

\textsuperscript{2} M. Montminy, manuscript in preparation.
cells (Fig. 4B). These results demonstrate that CREB is indeed an important epitope for recognition by αKK antibody. Under higher magnification, a punctate staining pattern was noted with αKK antisera in forskolin-stimulated cells, suggesting that CREB-CBP complexes are formed in discrete loci within the nucleus (Fig. 4C).

**DISCUSSION**

Our results illustrate a novel approach to the study of cellular signaling; to our knowledge, this is the first report documenting the formation of nuclear protein-protein complexes in situ. αKK antisera bind to residues in KID that undergo a conformational change following complex formation with KIX. The ability of αKK antisera to recognize full-length CREB-CBP complexes strongly supports the notion that this helical transition also occurs within the context of the full-length CREB protein. Structural transitions in transcription activators like CREB may therefore be integral to the process of recruiting the transcriptional machinery.

Other complex-specific antisera have been described, most notably against human immunodeficiency virus gp120 bound to its cellular receptor, CD4 (19–21). gp120 appears to undergo a conformational change, upon binding to CD4, that exposes an

**FIG. 2.** CREB-CBP-specific antisera recognizes a conformational change in CREB that accompanies complex formation. A, gel mobility shift assay of *C. elegans* phospho-(Ser$^{133}$) CREB (eP-CREB; lanes 1–5) and murine phospho-(Ser$^{133}$) CREM (Mouse P-CREM; lanes 6–9) using $^{32}$P-labeled CRE oligonucleotide. Reactions contained either eCREB or CREM plus KIX, αKK antisera, or α-phosphospecific CREB antisera indicated by plus signs over each lane. Bands in lanes 1 and 6 represent $^{32}$P-labeled phosphorylated eCREB-CRE and CREM-CRE complexes alone, respectively. B, sequence of homologous αA and αB regions in the KID domains of *C. elegans* CREB, mouse CREM, and rat CREB. Amino acid differences are shown in bold. C, gel mobility shift assay of wild-type and mutant (WT and M1, M2, and M3, respectively) GAL4-KID polypeptides using double-stranded GAL4-binding site oligonucleotide. Relative migration of GAL4-KID (P-KID), GAL4-KID-KIX (pKID-KIX), and αKK supershifted (Ab:KID-KIX) complexes bound to the $^{32}$P-labeled GAL4 oligonucleotide is shown. Addition of KIX or αKK antisera (αK-KID) to reactions is indicated by plus signs over each lane. D, ribbon diagram showing solution structure of KID-KIX complex. The KID domain is shown in yellow and the KIX domain is shown in cyan. The relative positions of residues important for αKK recognition (K136 and N139) on the αB helix are indicated. Reactions containing eP-CREB or Mouse P-CREM indicated by lines over lanes.

**FIG. 3.** Signal discrimination at the level of CREB-CBP complex formation. The effect of TPA and forskolin on signaling and target gene activation via CREB is shown. A, Western blot assay of total CREB and phospho-(Ser$^{133}$) CREB (P-CREB) levels in control (C) or treated D5 cells exposed to either forskolin (F) or TPA (T), as indicated, for 30 min. B, Northern blot assay of somatostatin (SOM) and tubulin (TUB) mRNA levels in control D5 cells (C) and in D5 cells treated with forskolin (F) or TPA (T) for 4 h.

**FIG. 4.** cAMP stimulates the formation of CREB-CBP complexes in situ. A, immunostaining of D5 cells following treatment with forskolin or TPA for 10 min. Staining with phospho-(Ser$^{133}$) CREB antisera (P-CREB) or with αKK antibody (CREB-CBP) is indicated. B, immunostaining of forskolin-treated (10 μM for 10 min.) CREB −/− and CREB +/+ fibroblasts from knockout and wild-type littermate mice, respectively. C, higher magnification of a forskolin-treated D5 cell stained with αKK antisera to show discreet CREB-CBP complexes.
epitope for recognition by complex-specific antisera. Indeed, a number of proteins appear to undergo structural changes upon complex formation with their cognate receptor or coactivator (22), suggesting that the development of complex-specific antisera may be generally useful for studies of cellular signaling.

CREB-CBP complexes appear to be formed at discrete regions within the nucleus. Although the constituents of these speckles are unknown aside from CREB and CBP, it is tempting to speculate that they may contain other components of the transcriptional apparatus. In this regard, CBP has been found to associate with RNA polymerase II holoenzyme complexes (23–25) as well as promyelocytic protein-containing nuclear bodies (26). Further analyses with appropriate antisera may reveal which of these complexes is recruited to CREB in response to cAMP but not other signaling pathways.

The ability of αKK antisera to distinguish between different signaling pathways demonstrates the utility of this reagent in monitoring cellular activity compared with phospho-(Ser133) CREB antisera. PhosphoCREB-specific antisera have been widely used, particularly in neuronal cells, to evaluate cellular responses to various stimuli. Our data suggest that some subset of these signals may not elicit a transcriptional response, at least via the same pathway as cAMP.

Phosphorylation of CREB in response to TPA is likely to be indirect and likely to involve extracellular signal-regulated kinases 1 and 2 and pp90RSK. Activation of the mitogen-activated protein kinase pathway may inhibit CREB-CBP complex formation by inducing phosphorylation of CREB at other inhibitory sites. In this regard, phosphorylation of CREB at Ser142 has been shown to block target gene activation in part by blocking CREB-CBP complex formation (9, 27). It will be of interest to determine the mechanism by which CREB discriminates between cAMP and other second messenger pathways.

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