Roles of Phosphorylation and Helix Propensity in the Binding of the KIX Domain of CREB-binding Protein by Constitutive (c-Myb) and Inducible (CREB) Activators*

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The general mechanism of transcriptional activation in eukaryotes involves interactions between DNA-bound activators, co-activators, and components of the basal transcription complex. Cyclic AMP response-element binding protein (CREB)-binding protein (CBP) is a general transcriptional co-activator that mediates interactions between transcription factors and the basal transcription machinery. To obtain insights into the mechanism by which the KIX domain of CBP can recognize the transactivation domains of many different transcription factors, we have used NMR and biochemical analyses to study the interactions of KIX with the transactivation domain from the constitutive activator c-Myb and with the kinase-inducible transactivation domain (KID) from CREB. NMR chemical shift mapping shows that both activation domains bind to the same surface of KIX. In the unbound state, both the phosphorylated KID and c-Myb activation domains are only partly structured, and binding to KIX is coupled with folding to form an amphipathic helix. Helix-destabilizing mutations significantly impair binding, whereas mutations that increase the intrinsic secondary structure content of the free phosphorylated KID peptide have only a small influence on binding affinity. Low affinity but specific binding of unphosphorylated KID to KIX was measured by ITC and was also observed in Western blot assays and by a fluorescence resonance energy transfer experiment in living cells. The large increase in the affinity for phosphorylated KID is due to favorable intermolecular interactions involving the phosphate moieties. After induction by phosphorylation, CREB is able to compete effectively with other transcriptional activators for binding to CBP.

The binding affinity of KIX for pKID is 20–50-fold higher than for c-Myb (6). This striking difference in affinity for KIX is consistent with the different kinetic profiles for the two activators. Whereas CREB is activated by phosphorylation within the KID domain, the activity of c-Myb is believed to be constitutive. Complex formation between pKID and KIX is enthalpy-driven, whereas c-Myb-KIX binding is driven by both enthalpy and entropy (6).

The apparent differences in the mode of binding of KIX to pKID and c-Myb prompted us to investigate the structural features of these complexes in order to obtain insights into the mechanisms by which the KIX domain of CBP recognizes nucleosome coherence; TFE, trifluoroethanol; ITC, isothermal titration calorimetry; PKA, protein kinase A; FRET, fluorescence resonance energy transfer.
merorous transcriptional activation domains. In particular, we were interested in evaluating the roles of phosphorylation and secondary structure formation in determining binding affinity for KIX and hence in the regulation of transcription. Our results confirm that a common hydrophobic binding interface of KIX is used to bind either pKID or c-Myb. In the absence of a phosphate group, KID gives rise to a low affinity complex with KIX that is not species-specific to the hydrophobic face. The intermolecular interactions of the phosphoserine in pKID provide substantial binding enthalpy, which, since it is not present in the unphosphorylated form of the protein, constitutes the factor that makes this interaction inducible. Our results also demonstrate that the binding region of the transactivation domains must be helical for tight binding to CBP and that mutations that destabilize the helix significantly impair binding.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The wild-type KIX domain (residues 586–672) of mouse CBP (identical amino acid sequence to human) was expressed in either unlabelled or uniformly ¹³C- and ¹⁵N-enriched forms in BL21(DE3) _Escherichia coli_ and purified to homogeneity by a previously described procedure (4). KIX 586–672 mutants Y558P, K626A, and Y650A were expressed and purified as glutathione S-transferase fusion proteins as described elsewhere (5). Glutathione S-transferase fusion proteins were then cleaved by thrombin, and KIX mutants (with an additional Gly-Ser peptide resulting from cleavage) were purified to homogeneity following the procedure used for wild-type KIX. The wild-type KID domain (residues 101–160 plus an N-terminal methionine) of mouse CREB (identical amino acid sequence to human) was expressed, purified, and phosphorylated as previously described (4). Shorter pKID/KID peptides (residues 119–147 (pKID29) or residues 129–149 (pKID21) of mouse CREB) and unlabelled Myb25 (residues 291–315 from _mouse_) were chemically synthesized using a Perseptive Biosystems peptide synthesizer (PerkinElmer Life Sciences) and purified to homogeneity by reverse-phase HPLC. Uniformly ¹³C- and ¹⁵N-labelled Myb25 291–315 were overexpressed in E. coli using a ubiquitin fusion protein system, which was a generous gift from Dr. Toshiyuki Kohno. The c-Myc 291–315 sequence was ligated to the ubiquitin sequence using a similar protocol to that described by Kohno et al. (9). Briefly, the decahistidine-tagged ubiquitin coding sequence was subcloned from plasmid pUBK9 (9) into a pET21d plasmid, digested with _NsiI_, blunted with T4 DNA polymerase, and digested by _BamHI_. The chemically synthesized oligonucleotide encoding c-Myc, followed by two stop codons and the _BamHI_ site, was amplified by PCR and digested by _BamHI_ and the 5′-end was phosphorylated by T4 polynucleotide kinase (New England Biolabs) and digested to the thermogram and subtraction of the blanks yielded a binding isotherm that was fit to a model of one-site interaction (ITC data analysis software in Origin 2.3 of MicroCal Inc.). Only _K_ₐ values are reported (together with S.D. from duplicate experiments), since _Δ_H_ values for the low affinity complexes have high uncertainties and were therefore not interpreted.

**Circular Dichroism**—Spectra were collected at 27 °C on the Aviv model 202 CD spectrometer using a path length of 2 mm. Samples contained 40–50 μM KID or Myb25 peptide in 5 mM potassium phosphate buffer (pH 7.0) and 0–40% (v/v) trifluoroethanol (TFE). The signal at 222 nm was normalized to give the molar ellipticity (θ) and used to determine the backbone random coil content by the CDPro program (16) using peptide concentrations determined by the isodichroic point observed at 202 nm was used to correct for minor concentration differences (16). The concentration of Myb25 was determined according to the stoichiometric ratio obtained from the curve fit. The concentration of KIX in the ITC cell was 50–165 μM with higher concentrations used for the lower affinity complexes, whereas the concentration of the peptide in the syringe was 12-fold over that of KIX. Typically, two injections of 5 μl were followed by 28 injections of 10 μl until a molar ratio of 2.5 was obtained. Integration of the thermogram and subtraction of the blanks yielded a binding isotherm that was fit to a model of one-site interaction (ITC data analysis software in Origin 2.3 of MicroCal Inc.). Only _K_ₐ values are reported (together with S.D. from duplicate experiments), since _Δ_H_ values for the low affinity complexes have high uncertainties and were therefore not interpreted.

**Western Blotting**—HEK293 cells were transfected with expression plasmids for KIX, KID, KID-KIX fusion protein, or S133A KID-KIX fusion protein. Western blotting from whole cell extracts using antisera against KID, KIX, and the KID-KIX complex was performed as previously described (19, 20). Phosphorylation of wild-type KID (but not S133A KID) on Ser-133 was confirmed with an anti-pKID antisera.

**Spectrofluorometric Fluorescence Resonance Energy Transfer (FRET) Measurement**—HEK293 cells (10⁴) were transfected for 24 h with plasmids encoding KID-EYFP and KIX-ECFP fusion proteins, as previously described (19, 20). The cells were resuspended in Fura-2 AM solution (Invitrogen) and placed in a glass cuvette with a stir bar. Emission spectra (460–540 nm) were recorded following excitation at 430 nm on a PTI spectrofluorometer.

## RESULTS

**Binding of c-Myb to KIX**—To map the site of c-Myb interaction on KIX, triple resonance NMR experiments were used to assign the backbone chemical shifts for ¹³C, ¹⁵N KIX complexed with a 25-mer peptide derived from the c-Myb activation domain (residues 291–315, hereafter called Myb25). Backbone amide chemical shift deviations of KIX upon complex formation (Fig. 1A) show that both Myb25 and pKID bind to the α₁₋α₃ interface of KIX, consistent with previous mutagenesis studies (6). The difference between experimental ¹³C chemical shifts and random coil values (termed secondary chemical shift) was

![NMR Spectroscopy](image-url)
used as a measure of backbone secondary structure (reviewed in Ref. 13) to evaluate the presence and extent of structural changes in KIX upon binding of c-Myb. As for pKID, Myb25 does not perturb the overall structure of KIX, since the Ca secondary shifts are indistinguishable from those previously determined for free KIX (21).

The KIX residues Tyr-658 and Lys-662, which interact with the phosphoserine in pKID, show larger chemical shifts for the complex with pKID (residues 119–147, hereafter called pKID29) than for the Myb25-KIX complex (Fig. 1A), suggesting that these residues contribute less to binding of Myb25, which lacks the phosphate. This is supported by biochemical studies showing that mutations of residues Tyr-658 or Lys-662 had a drastic effect on the affinity of pKID-KIX but only a minor effect on the affinity of c-Myb (6). Larger chemical shift perturbations are observed in the Myb25 complex for other KIX residues such as Tyr-650 and Leu-653 in the α3 helix and Leu-603, His-605, and Leu-607 residues in the α1 helix. The NMR data (Fig. 1A) are in good agreement with biochemical studies, in which mutations of residues Tyr-650 or Leu-653 had a larger effect on c-Myb binding than on pKID binding (6). Whether these differences result from direct contacts unique to one complex or subtle conformational changes in KIX remains to be explored.

The NMR data show unambiguously that Myb25 binds to the same α1–α3 face of KIX as the amphipathic αB helix of pKID (Fig. 2). In order to determine whether the structure of bound Myb25 is similar to that of pKID, the backbone resonances of 13C,15N Myb25, in the free and KIX-bound states, were assigned using triple resonance NMR experiments. As shown in Fig. 1B, the secondary chemical shifts of free Myb25 indicate that residues 295–309 populate a partially helical conformation. The helical content of this region, estimated by the magnitude of the secondary shifts, is 25–30%, which is consistent with CD results (6). Binding of KIX to Myb25 leads to a significant stabilization of the c-Myb helix, as evident in Fig. 1B. In particular, residues 295–306 in the center of Myb25 are completely helical in the bound state, whereas residues 292–294 and 307–311 show fraying of the helix. A similar fraying of the bound helix was observed in the interaction of pKID with KIX (8). Binding of both Myb25 and pKID to KIX involves a coupled folding event; the intermolecular interactions with KIX serve to stabilize the helical conformation of the two transcriptional activation domains.

**Binding of Unphosphorylated KID to KIX**—The binding of CREB to CBP in the absence of phosphorylation on Ser-133 has not been detected so far by *in vitro* techniques, including fluorescence polarization (22), glutathione S-transferase pull-down (23), and gel shift assays (3, 24, 25). This is puzzling in view of the important role of hydrophobic interactions of the αB helix of pKID in KIX binding (4) and of the structural similarities between binding of Myb25 and pKID. It is possible, however, that low affinity binding of unphosphorylated KID could escape the limited sensitivity of these assays. Isothermal titration calorimetry (ITC) was therefore used to measure the binding of unphosphorylated KID 101–160 (KID60), unphosphorylated KID 119–147 (KID29), and Myb25 to KIX. The results for KID60 and Myb25 are shown in Fig. 3A, and the $K_v$ values calculated for all of the peptides are reported in Table I (top). Our results demonstrate that unphosphorylated KID, either KID60 or KID29, will bind to KIX but with a 2-order of magnitude lower affinity than that of the phosphorylated state and a 7-fold lower affinity than that of the constitutive Myb25. The contribution of the phosphate to binding, calculated from these results to be a $\Delta G$ of $-3.0$ kcal/mol, agrees well with an NMR study that estimated the contribution of a dianionic phosphate group relative to a monoanionic one to be $-1.5$ kcal/mol (26).
To examine the specificity of complex formation between unphosphorylated KID and KIX, we employed an antisera that has been shown to bind specifically to the pKID-KIX complex (20). A fusion protein joining KID and KIX on a single polypeptide chain was prepared in order to achieve a high local concentration that would lead to detectable complex formation despite the low affinity of unphosphorylated KID for KIX. Following transfection of HEK293 cells with an expression plasmid encoding the fusion protein, Western blot analyses using the anti-complex-specific antibody recognized the KID-KIX fusion protein that lacks the phospho-acceptor Ser-133 (Fig. 3B, lane 3). The reduced intensity, relative to wild-type KID-KIX, is consistent with the lower affinity of KIX for unphosphorylated KID. The mutant S133A KID-KIX fusion protein was expressed at levels comparable with wild type as detected by both anti-KIX and anti-KID antibodies (Fig. 3B, lanes 7 and 11, respectively).

It has been shown previously that the anti-complex antibody recognizes specifically the $\alpha_B$ helix of KIX-bound KID (20). Our present results therefore indicate that the coil to helix transition in the $\alpha_B$ region of KID that accompanies binding to KIX is phosphorylation-independent. Thus, low affinity binding of unphosphorylated KID to KIX also results in folding of the $\alpha_B$ region into a helical structure.

**Significance of Helix Propensity in Binding to KIX**—The dependence of binding affinity on the propensity for helix in the free and bound states of the pKID and c-Myb peptides was examined by characterizing pKID29 peptides (Fig. 4A) with mutations at solvent-exposed positions in the $\alpha_A$ helix (residues 133–144). These mutations were designed, with the aid of the algorithm AGADIR (27), to increase (pKID29R135G-K136G) or decrease (pKID29K136G) the intrinsic helical content of the pKID29 peptide. It is important to note that the pKID29 peptides used in this study are derived from the minimal binding sequence 119–147, which has 4-fold lower affinity than the longer KID 101–160 (Table I), presumably due to less stable interactions of KIX with the shorter $\alpha_A$ helix.

CD spectra were recorded for the mutant pKID29 peptides in potassium phosphate buffer (5 mM, pH 7.0) to obtain insights into their relative helical propensities (i.e. their propensity to spontaneously form helical structure in the absence of stabilizing intermolecular interactions). The peptides were also titrated with TFE, a co-solvent that is known to induce and stabilize helical structure and that is often used to mimic helix formation upon binding to a receptor (28), to probe the relative stability of the helix formed upon binding of each peptide to the KIX domain. Previous CD and NMR studies of wild type pKID peptides showed that the $\alpha_B$ region spontaneously forms helical structure (50–60% population of helix) in aqueous solutions of the free peptide, whereas the intrinsic population of helix in the $\alpha_A$ region is very small (4, 6, 29). Thus, the helical contribution to the ellipticity at 222 nm ($\theta_{222} = 4000$ degrees cm$^2$ dmol$^{-1}$) (Fig. 4B) comes predominantly from the $\alpha_A$ region. The addition of TFE, up to 40%, to the wild-type peptide results in a decrease in $\theta_{222}$ to $-18000$ degrees cm$^2$ dmol$^{-1}$, corresponding to a change in helix content from $-10$ to $-53%$. This large change in ellipticity is due to stabilization of helical structure

**Table I**

| Peptide | Sequence | $K_d$ (phosphorylated) | $K_d$ (unphosphorylated) |
|---------|----------|------------------------|--------------------------|
| KID60  | CREB 101–160 | 0.7 ± 0.1              | 108 ± 5                  |
| KID29  | CREB 119–147 | 3.1 ± 0.6              | 120 ± 40                 |
| KID21  | CREB 129–149 (αB) | 80 ± 20               |                          |
| Myb25  | c-Myb 291–315 | 15 ± 5                |                          |

$K_d$ values are in μM. The errors represent S.D. from duplicate measurements.

**Fig. 3. Binding of KIX to unphosphorylated transcriptional activators.** A, ITC measurements of KIX binding to wild-type KID60, either phosphorylated (pKID60, gray circles) or unphosphorylated (KID60, open squares) and to Myb25 (open diamonds). KIX concentrations were 45 μM for the pKID60 titration, 150 μM for the corresponding experiment with unphosphorylated KID60, and 70 μM for the Myb25 titration. B, Western blots of HEK293 whole cell extracts transfected with either KID, KIX, KID-KIX fusion protein, or S133A KID-KIX fusion protein, as indicated above each lane. The blots were exposed to either anti-KIX complex antisera, anti-KID, or anti-KIX antisera, as indicated. Endogenous CREB is marked by an asterisk. Phosphorylation of wild-type KID and KID-KIX (but not S133A KID) on Ser-133 was confirmed with an anti-pKID antisera (data not shown).

**CD Spectra**

The CD spectra of the wild-type pKID29 and its mutants are shown in Fig. 4A. The spectra were recorded in the presence of 5% TFE to enhance the helical content of the peptides. The mutant pKID29 peptides showed a decrease in helical content compared to the wild-type peptide, with the exception of pKID29S133A, which showed an increase in helical content. The extent of this change in helical content was quantified by measuring the change in ellipticity at 222 nm ($\theta_{222}$) for each peptide in the absence and presence of TFE. The results are shown in Table II. It can be seen that the mutation of Ser-133 to Ala had the largest impact on the helical content of the pKID29 peptide, with a decrease in $\theta_{222}$ of 4000 degrees cm$^2$ dmol$^{-1}$.

**Table II**

| Peptide | $\theta_{222}$ (unphosphorylated) | $\theta_{222}$ (phosphorylated) |
|---------|---------------------------------|--------------------------------|
| Wild type | 3.1 ± 0.6                       | 110 ± 40                       |
| Y655F   | 110 ± 40                        | 5 ± 4                          |
| Y656A   | 21 ± 4                          | 39 ± 8                         |
| Y658A   | 39 ± 8                          | 0 ± 0                          |

$\theta_{222}$ values are in degrees cm$^2$ dmol$^{-1}$. The errors represent S.D. from duplicate measurements.
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in both the αA and αB regions in the presence of TFE (29). The TFE titration curves for phosphorylated pKID29 and for the unphosphorylated form of the peptide (KID29) are virtually identical, showing that phosphorylation does not significantly influence the helical propensity. Variations in θ_{222} at 0% TFE for the mutant pKID29 peptides (Fig. 4B) are in accord with the AGADIR predictions. The mutations in pKID29αA-5 and pKID29αA-6 result in negative values of θ_{222}, attributed to an increase in the intrinsic helicity of the αB region. Assuming that the contribution of αB helix to the CD spectrum of wild type pKID29 is negligible, an estimate of the helical population in the αB region is made from the changes in ellipticity (~10% helix in pKID29αA-5 and ~18% in pKID29αA-6). The maximal helical content in pKID29αA-6 at 40% TFE is the same as wild type peptide and is slightly lowered in pKID29αA-5. At 0% TFE, θ_{222} for pKID29αA-1 and pKID29αA-2 differs only slightly from that of wild type pKID29. 

The ellipticity of the Myb25 peptide (data not shown) is higher than that of the wild type KID29 peptides in aqueous solution, corresponding to the small but significant increase in intrinsic helicity of this peptide (6). As TFE is added, the ellipticity decreases to a minimum of about ~32,000 degrees cm² dmol⁻¹, corresponding to over 90% helix formation. This is consistent with the Cα chemical shift plot in Fig. 1B, which shows that the KIX-bound Myb25 peptide is completely helical between residues 295 and 307, without the kink between αA and αB that is seen for pKID (4, 8). This result implies that, although pKID and c-Myb bind in a similar site on KIX, the structure of the Myb peptide in the complex may have interesting differences from that of pKID.

Dissociation constants for binding of the mutant peptides to KIX were measured by ITC (Fig. 4C and Table I). The introduction of helix-destabilizing glycine residues in the αB helix of pKID29αA-1 and pKID29αA-2 leads to a very significant loss of binding affinity. The loss of affinity is 1 order of magnitude greater for the double mutant, although the CD data indicate that the two mutants have similar helical content in 40% TFE. These data unequivocally show the stability of the αB helix of pKID in the bound state is a major determinant of high affinity binding. In contrast, a double mutation to glycine in the αA helix of the peptide pKID29αA-2 reduced the affinity for KIX only 2-fold (Table I). These results suggest that formation of helical structure in the αA region contributes little to the overall binding affinity. However, N-terminal truncation of the peptide to completely eliminate the αA helix, in pKID21, results in a ~25-fold decrease in binding affinity. This can be naturally attributed to loss of Leu-128, which forms part of the hydrophobic interface between pKID and KIX in the NMR structure (4). Thus, our data clearly indicate that a helical conformation is necessary for αB residues to interact favorably with KIX but that helical structure is not essential in the αA region of pKID.

The importance of helix propensity for c-Myb interactions with KIX is also indicated by the inability of helix-disrupting c-Myb mutants L301P or E299P to bind KIX and activate transcription (6). It is likely that the loss of c-Myb activity observed in this functional assay reflects an inability to fold onto the KIX template.

The wild type pKID29, pKID29αA-5, and pKID29αB+6 peptides have comparable helicity in 40% TFE, and all bind KIX with high affinity (Fig. 3C, Table I). The substitution of five residues in pKID29αA-5 results in a slight decrease in binding affinity relative to wild type pKID29, although all of the sites of mutation are on the solvent-exposed surface of αB. The molecular basis for this is unknown, although we note that substitution of Lys-136 and Asn-139 with other helix-favoring side chains has been shown previously to make KIX binding less favorable (20). Substitution of Asp-140 in pKID29αA-5 by Glu, in the peptide pKID29αA-6, leads to a 3-fold increase in binding affinity (Table I). The increased binding affinity of pKID29αA-6 might reflect the increased helical population in the αB region of the free peptide, although at this stage we cannot rule out potential contributions from more favorable interactions between the Glu-140 side chain and Lys-606 in helix αB of KIX. What is clear, however, is that the dominant factor that determines the relative binding affinities of the various phosphorylated KID peptides is the stability of the αB helix in the KIX-bound state.

Taken together, these results indicate that although the propensity for helix formation in the free state of the transcriptional activator domain may have a small influence on its affinity for KIX, the stability of the helix formed upon binding (particularly the αB helix of pKID and, for c-Myb, the entire peptide) plays a major role in determining the overall affinity.

Significance of Phosphorylation in Binding of KID to KIX—Phosphorylation of KID on Ser-133 is required for binding to KIX and subsequent transactivation (2, 3). The contribution of the phosphate to the binding affinity of the inducible transcription...
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KID-EYFP, in which the PKA phosphorylation site is absent, resulted in a small but significant difference in the FRET signal from the control (Fig. 6). Integration of fluorescence loss and gain in the relevant wavelength ranges leads to estimates of basal binding to be ~15% of the inducible binding. Thus, binding of unphosphorylated KID to KIX can be detected in living cells as well as in vitro. Since these experiments require overexpression of KID and KIX in the cell, the functional significance of this interaction remains to be determined.

**DISCUSSION**

**Determinants of Binding to the KIX Hydrophobic Groove**—The results presented here highlight the significance of both secondary structure and phosphorylation for recognition of an inducible transcriptional activator by the KIX domain of the coactivator CBP and provide insights into the differences that are mandated in a constitutive transcriptional activator by the absence of the phosphate group. The combination of a relatively low binding constant for the constitutive activator and an even lower binding constant for the uninduced CREB suggests that competition between these factors (and others) for CBP may constitute a physiological control mechanism. According to our findings (schematically illustrated in Fig. 7A), the ability of the activator to form an amphipathic helix is necessary and sufficient to form a constitutive low affinity complex with KIX. Both the inducible pKID and constitutive c-Myb activation domains bind to the same face of KIX, formed by the \( \alpha_3 \) and \( \alpha_4 \) helices. Hydrophobic interactions comprise the major driving force for low affinity binding. The higher affinity of KIX for Myb25 relative to unphosphorylated KID suggests that the interactions at the common binding surface may be more extensive for Myb25. This is supported by the changes observed in KIX chemical shifts upon binding of Myb25, which, as shown in Fig. 2, extend significantly beyond the pKID contact surface.

The observed differences in the molecular interactions with pKID and c-Myb may be crucial to enable constitutive binding of c-Myb to KIX and at the same time to achieve low level basal interactions between CREB and KIX (Fig. 7). In accordance with this suggestion, Shaywitz et al. (30) showed that the magnitude of CREB-dependent transcriptional activity is determined by the strength of KID-KIX interaction. In particular, the L607F KIX mutant interacted more efficiently than wild type KIX with KID, leading to a significant increase in both basal and induced transcriptional activity of CREB but not c-Myb. Since Leu-607 is a core residue (4), these results imply that this KIX mutation may rearrange the \( \alpha_1-\alpha_2 \) interface in a way that specifically enhances binding of KID but not c-Myb. Forced overexpression of unphosphorylated KID led to detect-
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Fig. 7. Models for transcriptional activation by constitutive and inducible domains. A, schematic diagram representation of KIX recognition by transcription activation domains. The low affinity constitutive binding of c-Myb to KIX is dominated by hydrophobic interactions (red dashed lines) between the stabilized amphipathic helix of c-Myb and the α1-α2 interface of KIX. The basal binding of CREB has lower affinity and requires a larger degree of secondary structure formation. Phosphorylation of CREB on Ser-133 by PKA leads to enhancement of binding affinity as a result of additional interactions between the phosphate group and Tyr-658 and Lys-662 of KIX (green lines). B, model describing expected levels of gene-specific transcription by c-Myb (red) and CREB (green), modulated by competition for limiting amounts of CBP. Expression of c-Myb is predicted to lower basal CREB-mediated transcription (region 1), whereas phosphorylation by PKA would increase the affinity of CREB to KIX, allowing it to compete efficiently with c-Myb and other constitutive activators for binding to CBP (region 2). pCREB, phosphorylated CREB.

Possible Roles for Induced Fit in CREB Signaling—Our data show that the stability of the αh helix of pKID in the KIX-bound state is a critical determinant of binding affinity; destabilization of this helix through glycine mutagenesis diminishes binding affinity by 1–2 orders of magnitude. In contrast, changes in the intrinsic population of the αh helix in the unbound state have only a modest influence on the thermodynamics of complex formation. The evolutionary conservation of a sequence that is able to form stable helical structure only upon binding to its target raises interesting questions regarding a possible physiological role for the unfolded state of unbound KID. It is possible that this intrinsic lack of structure may confer a functional advantage for CREB by allowing it to serve as a ligand for several different proteins. In particular, the catalytic subunit of PKA has been shown to bind a peptide inhibitor in an extended conformation (31). Therefore, the absence of strong secondary structural propensities in the unbound state of KID may facilitate interaction with and phosphorylation by PKA, which in turn promotes high affinity binding to CBP through the favorable hydrogen bonding interactions formed by the phosphoryl group (4). In addition, a theoretical study (32) suggested that the speed of molecular recognition can be enhanced by having folding (necessary for the required specificity) coupled to binding rather than occurring before. Bienkiewicz et al. (33) have recently demonstrated for the p27Kip1-cyclin A-Cdk2 system that binding-coupled folding has an advantage over a preformed structure by acceleration of molecular recognition. Therefore, apart from the thermodynamic differences, kinetic discrimination between the constitutive activator c-Myb and kinase-inducible activation domain of CREB is possible due to the larger degree of intrinsic disorder in the αh helix region of unbound pKID.

Conclusion—Our results provide new insights into the mechanism of recognition of various transcriptional activator domains by the highly conserved KIX domain of CBP and p300 and illustrate the structural and thermodynamic basis for constitutive activation by c-Myb and inducible activation by CREB. We suggest that the minimal requirement for interaction with the hydrophobic groove of KIX is binding-coupled stabilization of an amphipathic helix, which we have shown to be sufficient for the generation of a low affinity complex. Modulation of that complementarity enables the KIX domain to distinguish between the constitutive c-Myb that activates transcription and the unphosphorylated form of CREB that should not activate transcription. Induction of CREB by phosphorylation converts the low affinity complex into a specific high affinity complex mainly via formation of additional intermolecular interactions.

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