

Cooperativity in Transcription Factor Binding to the Coactivator CREB-binding Protein (CBP)

THE MIXED LINEAGE LEUKEMIA PROTEIN (MLL) ACTIVATION DOMAIN BINDS TO AN ALLOSTERIC SITE ON THE KIX DOMAIN*

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The interactions between cAMP-response element-binding protein (CREB)-binding protein (CBP) and gene-specific transcription factors play an important role in activation of transcription from numerous genes. Cooperative interactions between CBP and multiple transcriptional activators may provide a mechanism for synergistic increases in transcriptional activation. Here we report the characterization of ternary complexes formed by the KIX domain of CBP and the transactivation domain of the trithorax group protein mixed lineage leukemia protein (MLL), together with either the phosphorylated kinase-inducible domain (pKID) of CREB or the activation domain from c-Myb. Isothermal titration calorimetry experiments show that KIX in complex with the MLL activation domain binds the c-Myb activation domain and pKID domain with 2-fold higher affinity than does the KIX domain alone. Thus, the activation domains of Myb and MLL or of pKID and MLL bind cooperatively to KIX. The thermodynamics of these interactions imply different mechanisms of binding cooperativity for the two ternary complexes; the KIX-MLL-pKID complex is stabilized by entropy increases, whereas the enhancement of Myb binding in the presence of the MLL activation domain is due to more favorable enthalpy. NMR experiments show that the MLL-binding site on KIX is distinct from the surface that binds the pKID and c-Myb activation domains. Our data indicate that KIX can directly mediate cooperative interactions between pairs of transcriptional regulatory proteins. In the case of MLL and c-Myb, both proteins are involved in proliferation of hematopoietic cells and leukemogenesis, and synergistic interactions mediated by CBP may play a functional role.

ficity, timing, and degree of transcriptional activation depend not only on which proteins form this complex but also on how they interact with each other. At the simplest level, direct interactions involved in complex formation provide a mechanism for recruiting specific functionalities to a promoter sequence. However, in some cases, binding events also give rise to allosteric effects on binding or enzymatic activity, providing another means by which transcriptional activity can be modulated.

A transcriptional coactivator that may be allosterically regulated by simultaneous interactions with more than one target protein is the cAMP-response element (CREB)¹-binding protein (CBP), as well as its paralog p300. In addition to its catalytic histone acetyltransferase domain, CBP is composed of several protein-binding modules that serve to bridge gene-specific transcription factors with components of the basal transcription complex (2). CBP domains such as KIX, CH1, and CH3 recognize a diverse range of protein sequences that in some cases appear to share a general structural motif in the bound state. For example, the KIX domain binds an amphipathic helix formed by either the phosphorylated kinase-inducible domain (pKID) of CREB or the transactivation domain of c-Myb (3, 4). Although both of these ligands bind to the shallow hydrophobic groove formed between KIX helices α_1 and α_3 , the high affinity of CREB for CBP is mediated by critical interactions involving the pKID phosphoryl group and is therefore inducible, whereas the c-Myb activation domain binds constitutively with somewhat lower affinity (5, 6).

It has been proposed that CBP-mediated transcriptional activation can be enhanced by cooperative interactions between CBP and more than one DNA-bound transcriptional activator (7–9). Since the nuclear concentration of CBP is thought to be limiting (10–12), the presence of multiple protein-binding domains on CBP, together with the high affinity of CBP for the activators involved, should sequester a larger proportion of CBP to specific promoter sites and thereby provide a mechanism for transcriptional synergy by multiple activators. There are a number of cases where synergistic increases in transcription have been shown to occur when multiple CBP-binding activators bind to the same gene promoter. Examples include cooperative transcriptional activation by CREB and the serum response factor (13, 14) as well as enhancement of c-Myb transcriptional activation by core binding factor (15, 16), CCAAT/

Transcriptional activation is mediated by large protein complexes assembled on target gene promoter regions. These complexes contain activators and coactivators of transcription as well as elements of the basal transcription machinery (1). The speci-

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¹ The abbreviations used are: CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; MLL, mixed lineage leukemia protein; pCREB, phosphorylated CREB; pKID, phosphorylated kinase-inducible activation domain; TAD, transactivation domain; HSQC, heteronuclear single quantum coherence; ITC, isothermal titration calorimetry; Ci, *cubitus interruptus* gene product; Gli, glioblastoma gene product.

enhancer-binding protein β (17), or Ets-1 (18). These examples strongly support a role for CBP in transcriptional synergy. However, it has not yet been established that CBP itself can cooperatively interact with multiple activators to synergistically enhance transcription.

Whereas the array of protein-binding domains contained in CBP suggests that cooperative interactions could involve more than one domain, results from a number of studies indicate that the CBP KIX domain alone can form a ternary complex with various transcription factors (19–23). One of the most compelling examples of this was provided by Ernst *et al.* (24), who characterized the interaction between KIX and the activation domain of mixed lineage leukemia (MLL) protein (also called Htrx, ALL-1, and HRX). MLL binding was shown to stabilize the interaction between KIX and phosphorylated CREB, and residues critical to this interaction were localized to a short region within the MLL activation domain. MLL, a homologue of the *Drosophila* trithorax (Trx) protein important for the maintenance of homeobox (Hox) gene expression (25, 26), is widely expressed (27, 28), acts antagonistically with *Polycomb* group (PcG) repressors of transcription (29), and is crucial for the definition of segment identity in early embryogenesis (30). The observation that MLL can interact with the KIX domain provided the first indication that CBP may also play a role in transcriptional activation maintained by MLL and that CREB and MLL may act together to activate transcription of genes that have yet to be identified.

Since cooperative binding to CBP may promote synergism in transcriptional activation, we investigated ternary complex formation involving KIX and the MLL activation domain using isothermal titration calorimetry (ITC) and NMR. The results show that KIX can cooperatively bind a peptide derived from the MLL transactivation domain and either the pKID domain of CREB or the c-Myb transactivation domain. MLL binds to a distinct site on KIX that is remote from the hydrophobic groove to which pKID or c-Myb bind. Overall, our data establish that the KIX domain can directly mediate cooperative ternary complex formation, providing a potential mechanism by which transcriptional synergy can be mediated by CBP.

EXPERIMENTAL PROCEDURES

Sample Preparation—All peptides under 40 residues were prepared by standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-based solid phase peptide synthesis using a Pioneer Peptide Synthesis System (Perspective Biosystems). pKID34 (CREB residues 116–149) is phosphorylated at Ser-133. To avoid potential dimer formation by MLL19-(2840–2858) and MLL31-(2840–2869), Cys-2841 was substituted by Ala; this was considered unlikely to interfere with binding assays, since residues N-terminal to 2844 are not involved in transactivation (31). All three peptides were amidated at the C terminus and, except for MLL31, acetylated at the N terminus. MLL31 includes a nonnative N-terminal serine residue. All peptides were purified to homogeneity by reverse phase HPLC.

The minimal MLL transactivation domain (TAD; residues 2813–2887) was amplified by PCR from a human liver cDNA library (Mobicat catalog no. GNGL09) and subcloned into a KIX co-expression vector derived from a single pET22b-derived vector with two cloning and ribosomal binding sites (32). The TAD was purified with a HitrapQ column (Amersham Biosciences) followed by reverse phase HPLC. Myb25-(291–315) and ^{15}N , ^{13}C -labeled KIX-(586–672) were prepared as previously described (6, 33). Protein masses were confirmed using matrix-assisted laser desorption/ionization mass spectroscopy. Concentrations were determined either by amino acid analysis performed in triplicate or absorbance at 280 nm.

ITC—Protein samples were prepared in 50 mM Tris, pH 7.0, 50 mM NaCl and were filtered and degassed. Titration of pKID34, Myb25, or MLL19 (~0.6 mM) into KIX (50 μM) was performed at 27 °C using an MCS titration calorimeter from MicroCal Inc. Two injections of 5 μl were followed by 28 injections of 10 μl until a molar ratio of ~2.5 peptide/protein was obtained. Integration of the thermogram and subtraction of the blanks yielded a binding isotherm that fit best to a model

of one-site interaction (34), using the ITC data analysis software Origin 2.3 (MicroCal). A nonlinear least-squares curve-fitting algorithm was used to determine the stoichiometric ratio, the dissociation constant (K_d), and the change in enthalpy of the interaction. The change in entropy was then indirectly determined according to Equation 1,

$$-RT \ln (1/K_d) = \Delta G = \Delta H - T\Delta S \quad (\text{Eq. 1})$$

where R is the gas constant, T is the absolute temperature, and ΔG , ΔH , and ΔS are the free energy, enthalpy, and entropy changes, from the free to bound states. The stoichiometric ratio obtained from the curve fit was 1:1 to within 5% error. All ITC experiments were performed in duplicate.

Ternary complex formation was monitored with a larger than 2-fold molar excess of MLL19 to KIX, ensuring that >90% of KIX was bound to MLL19 before titration of pKID34 or Myb25. The thermodynamics of MLL19 binding to KIX was also measured with >95% of KIX bound to pKID34 or >97% of KIX bound to Myb25. As predicted by pairwise coupling theory (reviewed in Ref. 63), the total free energy of ternary complex formation did not depend on the order of ligand added and was therefore consistent with Equation 2,

$$\Delta G_{\text{KIX-KIX/MLL}} + \Delta G_{\text{KIX/MLL-KIX/MLL/pKID}} = \Delta G_{\text{KIX-KIX/pKID}} + \Delta G_{\text{KIX/pKID-KIX/pKID/MLL}} \quad (\text{Eq. 2})$$

where $\Delta G_{x,y}$ is the free energy going from state x to state y . Titration of MLL19 (0.6 mM) into Myb25 or pKID34 (50 μM), in the absence of KIX, did not show any evidence of binding (data not shown).

NMR Spectroscopy—NMR spectra processed by NMRPipe (36) of 0.5–0.8 mM ^{15}N , ^{13}C -labeled KIX were acquired on Bruker 500- and 600-MHz spectrometers at 300 K in 20 mM Tris acetate, pH 5.5, 50 mM sodium chloride, 0.2% (w/v) sodium azide, and 10% D_2O . Unlabeled MLL19 (binary complex) or MLL31 and Myb25 (ternary complex) peptides were included at a 3-fold molar excess over KIX. Complete backbone ^{15}N , ^1H , $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ chemical shift assignments (excluding the amide resonance from the N-terminal Gly-586) were obtained for each complex by NMRview analysis (37) of HNCACB and CBCA-(CO)NH spectra (38) and by comparison with chemical shift assignments from free (33) and Myb25-bound (6) KIX. ^1H , ^{13}C , and ^{15}N chemical shifts were referenced using a 2,2-dimethyl-2-silapentane-5-sulfonate standard, and $\text{C}\alpha$ secondary shifts from peptide-derived random coil values (39) were calculated using NMRview.

RESULTS

Binding of MLL to KIX Measured by ITC—To determine the thermodynamic parameters of MLL binding to the KIX domain of CBP, a 19-mer peptide that encompasses residues 2840–2858 (MLL19) was synthesized from the MLL activation domain. This peptide includes the core region of the MLL minimal transactivation domain (residues 2844–2854) that was shown to be important for interaction with the KIX domain as well as for transactivation (24, 31). Titration of MLL19 into KIX monitored by ITC revealed a high binding affinity (dissociation constant $K_d = 2.8 \pm 0.4 \mu\text{M}$) (Fig. 1A and Table I). ITC measurements of KIX binding affinities using a longer MLL construct (residues 2813–2887) gave similar results, indicating that the minimal region required for KIX binding is contained within the MLL19 peptide.

KIX Binds Cooperatively to pKID and MLL—The apparent cooperativity of MLL and CREB binding to KIX (24) encouraged us to investigate ternary complex formation using the pKID domain of CREB. ITC experiments revealed that a phosphorylated peptide encompassing CREB residues 116–149 (pKID34) binds to KIX with high affinity in the presence of nearly saturating amounts of MLL19. Moreover, the strength of this interaction was enhanced by MLL19, since the K_d for pKID34 binding to the KIX-MLL19 complex showed a 2-fold decrease over binding to KIX alone, from 1.30 ± 0.02 to $0.65 \pm 0.05 \mu\text{M}$ (Fig. 1B and Table I). As expected for cooperative binding systems in thermodynamic equilibrium, pKID34 also increased the affinity of MLL19 binding to KIX by ~2-fold (Table I). Direct binding between the minimal activation domain of MLL, and the pKID activation domain was not observed either by ITC or NMR (data not shown),

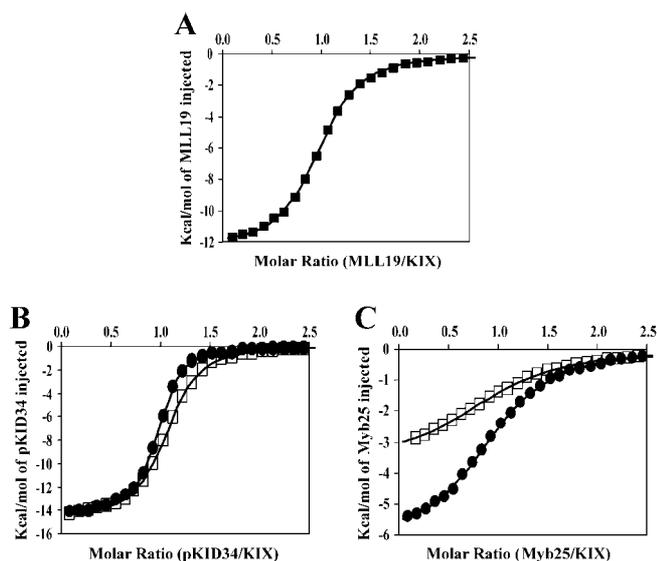


FIG. 1. Cooperative KIX binding by MLL19 and pKID34 or Myb25. Representative binding isotherms obtained for the titration of 0.6 mM solutions of MLL19 (A) pKID34 (B), or Myb25 (C) into 50 μ M KIX in the presence (circles) or absence (squares) of MLL19. The heats (in kcal/mol injectant) associated with ternary complex formation were measured with a larger than 2-fold molar excess of MLL19 over KIX. According to the dissociation constant measured for the MLL19-KIX complex ($2.8 \pm 0.4 \mu$ M), >90% of KIX should be bound to MLL19 under these conditions, as was confirmed by the ITC titration profile.

indicating that cooperativity is mediated by direct interactions with the KIX domain.

Since the enthalpy of complex formation can be directly obtained by fitting the binding isotherm to a one-site model of interaction, the energetic origin for cooperative KIX binding was examined by comparing the enthalpy of binding at a single site (*i.e.* the pKID- or MLL-binding site) in the presence or absence of ligand occupying the second site (Table I). In general, we found that the favorable enthalpy associated with either the MLL19 or pKID34 interaction with KIX was lower in the presence of the complementary ligand (pKID34 or MLL19, respectively). Consequently, the driving force for binding cooperativity arises from a decrease in the entropic penalty of complex formation, which was larger than the enthalpy change.

Cooperative Ternary Complex Formation by KIX, MLL, and c-Myb—In addition to its role in embryonic development, MLL is critical for the production of normal blood cells (32). Interestingly, the KIX-binding protein c-Myb is almost exclusively expressed in hematopoietic precursor cells (41, 42), where, like MLL, it is essential for proliferation (43–45). Since both MLL and c-Myb can bind KIX and are expressed at the same developmental stage of immature blood cells, we investigated whether these proteins can either compete for CBP binding or instead interact cooperatively with CBP.

To address this question, ITC experiments monitoring KIX binding in the presence and absence of MLL19 were performed with a peptide encompassing c-Myb residues 291–315 (Myb25) that includes the minimal trans-activation domain (3). As shown in Fig. 1C, Myb25 binds with high affinity to MLL19-saturated KIX ($K_d = 4 \pm 1 \mu$ M). Comparison of dissociation constants obtained for Myb25 binding to KIX in the presence and absence of MLL19 show that these peptides do not compete for KIX but rather bind cooperatively (Fig. 1C and Table I). Similar to the KIX binding cooperativity observed for MLL19 and pKID, MLL19 and Myb25 affinity for KIX is enhanced by \sim 2-fold in the presence of the complementary KIX ligand.

In contrast to the pKID34-KIX interaction, where binding cooperativity with MLL19 results from a reduction in the en-

tropic requirement for complex formation, the mechanism for binding synergism with Myb25 and MLL19 is enthalpic (Table I). In this case, entropy changes do not drive ternary complex formation, since the favorable entropy of Myb25 binding is reduced by MLL19, whereas the unfavorable entropy of MLL19 binding increases in the presence of Myb25. Instead, occupation of one binding site on KIX increases the favorable enthalpy associated with the second binding event, overcoming the unfavorable change in entropy.

Chemical Shift Mapping of the MLL Binding Site on KIX—To localize the MLL binding site on KIX, backbone chemical shift assignments were obtained for 15 N, 13 C-labeled KIX saturated with MLL19 using standard triple resonance NMR experiments. As shown in Fig. 2, the 1 H- 15 N HSQC spectrum of free KIX exhibits the chemical shift dispersion characteristic of a folded, monomeric domain (33). However, upon the addition of MLL19, a number of peaks in the KIX spectrum disappeared with concomitant appearance of peaks at different chemical shift positions (Fig. 2, red). Since only a subset of the resonances undergoes significant changes during the course of the titration, these spectral differences are indicative of site-specific binding of MLL19 to KIX. Large chemical shift changes occurring upon MLL19 binding were localized to the amino-terminal region of the α_2 helix of KIX, as well as the linker that connects helices α_1 and α_2 , including the short 3_{10} helix termed G_2 (Fig. 3A). The proposed MLL-binding surface of KIX is remote from the pKID-binding hydrophobic groove formed by helices α_1 and α_3 (Fig. 4A).

MLL and c-Myb Bind to Distinct Sites on KIX—To determine whether Myb25 binds to the hydrophobic groove of KIX in the presence of saturating amounts of MLL, backbone chemical shifts were assigned for 15 N, 13 C-labeled KIX bound to Myb25 and a slightly longer MLL polypeptide (MLL31; see “Experimental Procedures”). Fig. 3B shows that the largest backbone amide chemical shift perturbations that occur upon Myb25 binding to KIX-MLL31 localize to KIX helices α_1 and α_3 , the resonances of which were largely unaffected by MLL binding (Fig. 3A). Mapping these chemical shift perturbations to the molecular surface of KIX (Fig. 4B) shows clearly that Myb25 binds to the same surface as pKID, since the largest changes localize to a deep pocket within the hydrophobic groove and Tyr-658 on helix 3, as was previously observed for the binary Myb25-KIX complex (6). These results are in accordance with the thermodynamic data (Fig. 1C and Table I), showing that binding of either Myb25 or MLL to KIX increases the affinity of the second ligand for KIX. Therefore, the KIX domain of CBP is capable of mediating cooperativity between c-Myb and MLL via direct interactions. KIX amide chemical shift differences that occur upon MLL binding and subsequent c-Myb binding (Fig. 3) show that residues 611 and 612, immediately following the α_1 helix, sense both binding events, suggesting that this region could be an allosteric switch mediating binding cooperativity between this pair of ligands.

KIX Backbone Conformation in the MLL Complex—To determine whether the amide chemical shift changes arising from MLL19 binding reflect some alteration of KIX structure, secondary $C\alpha$ chemical shifts (defined as the difference between experimental and average random coil values (40)) were calculated for KIX in the presence and absence of MLL, since secondary shifts primarily reflect local backbone conformations. There is excellent agreement between secondary structure elements determined in the NMR structure of pKID-bound KIX and those suggested by secondary $C\alpha$ chemical shifts for both pKID-bound and free KIX (33). The secondary $C\alpha$ shifts of MLL-bound and free KIX (Fig. 5) as well as pKID-bound KIX (33) are very similar overall, confirming that the backbone

TABLE I
Thermodynamic parameters of binary and ternary complex formation

K_d and ΔH values were directly obtained by a fitting procedure to the ITC data, whereas ΔS was indirectly determined as described under "Experimental Procedures."

Ligand	Binding to	K_d^a	Energy			Energy difference ^d	
			ΔG	ΔH^b	$-T\Delta S^c$	$\Delta\Delta H$	$-T\Delta\Delta S$
		μM		$kcal/mol$			
Myb25	KIX	10 ± 2	-6.9	-3.5	-3.4		
Myb25	KIX-MLL19	4 ± 1	-7.4	-6.1	-1.3	-2.6	2.1
MLL19	KIX	2.8 ± 0.4	-7.7	-12.2	4.5		
MLL19	KIX-Myb25	1.7 ± 0.1	-7.9	-14.7	6.8	-2.5	2.3
pKID34	KIX	1.30 ± 0.02	-8.1	-14.7	6.6		
pKID34	KIX-MLL19	0.65 ± 0.05	-8.6	-14.4	5.9	0.3	-0.7
MLL19	KIX	2.8 ± 0.4	-7.7	-12.2	4.5		
MLL19	KIX-pKID34	1.5 ± 0.2	-8.1	-11.1	3.1	1.1	-1.4

^a The errors represent S.D. from duplicate measurements.

^b The reproducibility of ΔH for duplicate measurements was typically 0.2 kcal/mol or better.

^c The reproducibility of $-T\Delta S$ for duplicate measurements was approximately 0.3 kcal/mol.

^d Difference in energy between the indicated ternary complex and the preceding binary complex.

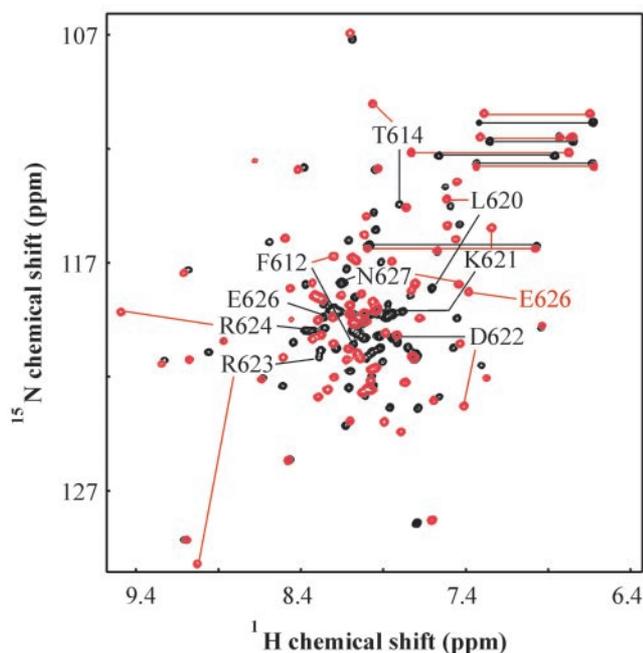


FIG. 2. **MLL binding to KIX monitored by NMR.** Superposition of 1H - ^{15}N HSQC spectra from MLL19-bound ^{15}N -labeled KIX (red) onto that of the free form (black). Residues showing significant backbone amide chemical shift changes are labeled. Horizontal lines connect peaks originating from Gln or Asn side chain amide proton pairs.

structure of KIX is not perturbed upon binary complex formation. Examination of secondary $C\alpha$ shifts in the ternary complex (data not shown) confirms that KIX backbone structure is also maintained when bound to both MLL and Myb25.

Although the trends in $C\alpha$ secondary shifts are preserved between MLL19-bound KIX and free KIX (Fig. 5), some residues that show amide chemical shift perturbations upon MLL19 binding also exhibit some difference in secondary $C\alpha$ shifts (e.g. Phe-612 and Arg-623). This may reflect changes in the local chemical environment resulting, for example, from proximity to charged or aromatic MLL side chains. In contrast, over the length of the G_2 3_{10} helix (residues 617–620) (Fig. 6A), $C\alpha$ secondary shifts increased uniformly by ~ 1 ppm, although amide chemical shift deviations were not as dramatic in this region (compare Figs. 3A and 5). This change indicates an increase in helical structure for the G_2 segment. It has been suggested that the α_1 - α_2 linker in KIX is intrinsically flexible, since the resonances from these residues are narrow and poorly dispersed in the free KIX spectrum (Fig. 2) as well as in the

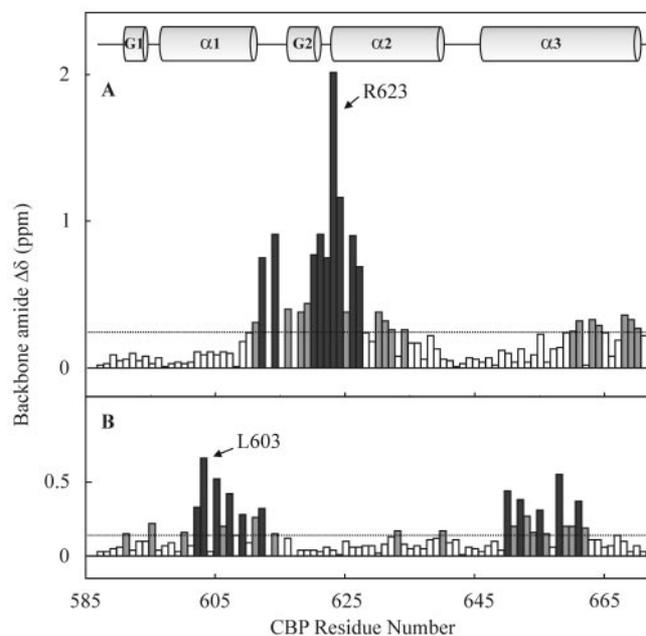


FIG. 3. **Distinct interfaces for MLL and c-Myb binding to KIX.** Average chemical shift deviations ($\Delta\delta$) for KIX backbone amide 1H and ^{15}N atoms that occur upon binding of MLL19 to KIX (A) or Myb25 to the KIX-MLL31 complex (B) were calculated according to the formula $\Delta\delta = \sqrt{(\Delta\delta_{HN})^2 + (\Delta\delta_N/5)^2}$, where $\Delta\delta_{HN}$ and $\Delta\delta_N$ are the amide proton and nitrogen chemical shift differences, respectively. The horizontal line in each graph denotes the average chemical shift difference (0.24 ppm in A, 0.14 ppm in B). The black bars indicate residues that exhibit chemical shift deviations exceeding the average difference by more than 1 S.D. value, whereas gray bars denote smaller shift differences above the average. The location of secondary structural elements in the NMR structure of KIX in complex with pKID (5, 38) is schematically depicted at the top.

pKID-bound spectrum (5). Moreover, in the ensemble of NMR structures determined for KIX in complex with pKID (5), this region is not as well defined as the core structure. The $C\alpha$ secondary shifts suggest that the G_2 helix may be stabilized by MLL19 binding, potentially influencing the ability of KIX to bind to other ligands such as CREB. The potential reduction in KIX conformational flexibility upon MLL binding is consistent with the thermodynamic data showing a reduction in the entropic barrier to ternary complex formation (Table I). Taken together, these data support a model where the first binding event stabilizes a KIX conformation favoring the second binding event, although additional structural information will be required to more precisely determine the origin of the observed KIX binding cooperativity.

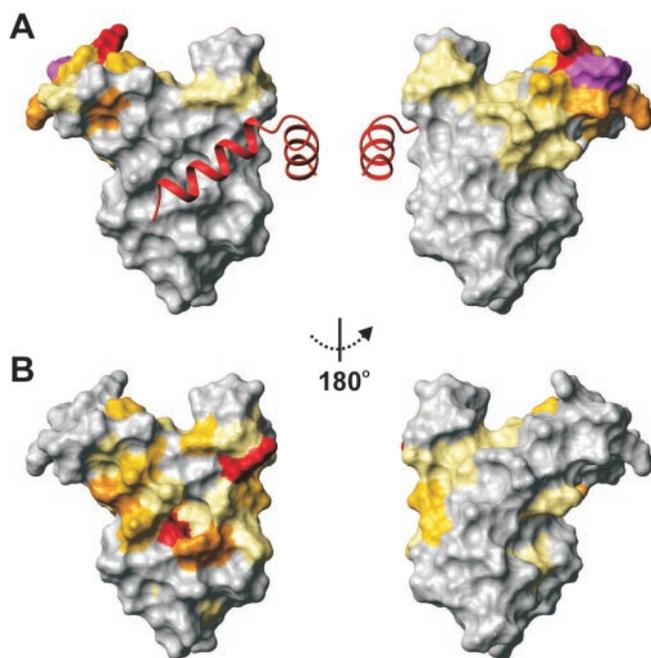


FIG. 4. Distinct binding site for MLL on KIX. *A*, backbone amide chemical shift deviations ($\Delta\delta$) for KIX resulting from binary complex formation with MLL19 are color-mapped onto the surface representation of the minimized mean KIX structure (5). The pKID polypeptide (red ribbon) is included to illustrate the distinct binding surfaces for pKID and MLL, although pKID was not present in the chemical shift mapping experiment represented here. Two views differing by 180° rotations about the vertical axis are shown. Yellow, gold, orange, red, and magenta surfaces indicate residues that exhibit a $\Delta\delta$ exceeding the average shift difference (0.24 ppm) by 1, 2, 3, 4, and 6 S.D. values ($\sigma = 0.31$ ppm), respectively. *B*, chemical shift differences ($\Delta\delta$) arising from Myb25 binding to the KIX-MLL31 complex, color-mapped onto the KIX structure as described for *A*. Average $\Delta\delta = 0.14$ ppm, $\sigma = 0.13$ ppm.

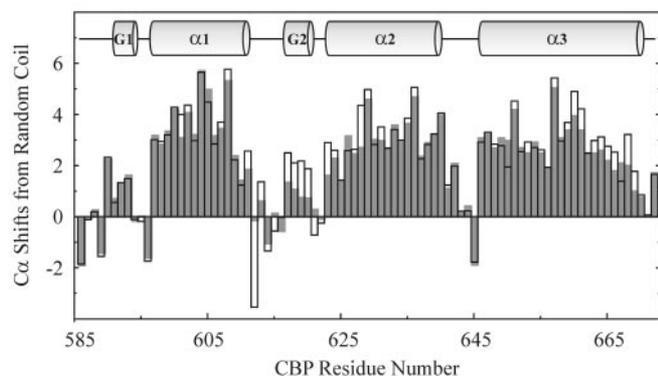


FIG. 5. KIX backbone structure is not significantly perturbed upon MLL19 binding. Shown are secondary $C\alpha$ chemical shifts, representing the difference between experimental $C\alpha$ chemical shifts and published random coil values (39), for MLL19-bound KIX (open bars) and free KIX (gray bars). Regions showing positive secondary $C\alpha$ chemical shifts indicate a helical conformation. Secondary structure elements in the NMR structure of KIX bound to pKID are depicted at the top (5, 38).

DISCUSSION

A Distinct Binding Site on KIX for MLL—The results from this study provide the first conclusive evidence that the KIX domain of CBP can participate in direct and simultaneous interactions with more than one protein. The amide chemical shift perturbations resulting from MLL19 binding (Fig. 3A) mapped onto the previously determined NMR structure of KIX illustrate that the largest differences localize to a region of KIX that is remote from the pKID-binding hydrophobic groove formed by helices α_1 and α_3 (Fig. 4A). Some residues showing large chemical shift deviations

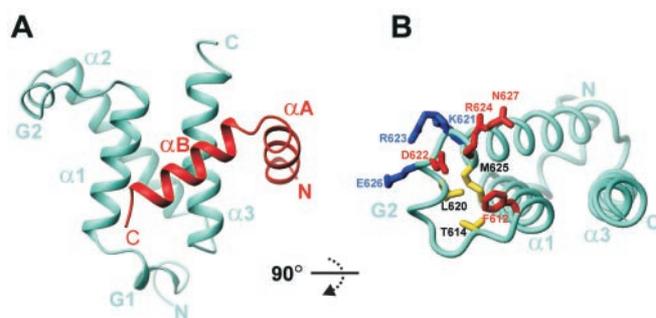


FIG. 6. Conserved KIX residues at the MLL-binding site. *A*, ribbon diagram of KIX (cyan) in complex with pKID (red), as determined by solution NMR (Protein Data Bank accession code 1kdx (5)). *B*, ribbon diagram of KIX highlighting residues that exhibit the largest amide chemical shift deviations upon MLL-binding by side chain stick representations. This view differs from that in *A* by a 90° rotation about the horizontal axis. Red side chains denote conserved, solvent-exposed residues; blue indicates nonconserved, solvent-exposed residues; and yellow shows conserved, buried side chains.

tions upon MLL binding, namely Phe-612, Asp-622, Arg-624, and Asn-627, are solvent-exposed (Fig. 6B, red side chains) and are almost completely conserved in all known KIX sequences (5). Given that these residues are not involved in pKID binding, it is likely that the side chains of these residues participate in direct interactions with MLL. Conversely, other solvent-exposed residues in this region, such as Lys-621, Arg-623, and Glu-626 (Fig. 6B, blue side chains), are not well conserved. The large amide chemical shift differences observed for these residues could reflect a role for backbone atoms in the interaction or may simply arise from proximity of these residues to the MLL-binding site. Other conserved residues showing chemical shift sensitivity to MLL binding are Leu-620 from the G_2 helix, Met-625 from α_2 helix, and the loop residue Thr-614 (Fig. 6B, yellow side chains). These amino acids directly interact with each other in the KIX structure to form a central part of the “secondary hydrophobic core” that was previously described for this region (5). A high level of sequence conservation for KIX secondary core residues corroborates its importance as a scaffold for the MLL binding site.

Thermodynamic Driving Force for Ternary Complex Formation Differs for pKID and c-Myb—The ITC results show unambiguously that the cooperative formation of a ternary complex between KIX, pKID, and MLL is mediated by a decrease in the entropic penalty of pKID-KIX complex formation. These energetic differences suggest that cooperativity is achieved by the creation of interactions unique to the pKID-KIX-MLL ternary complex. We suggest that these interactions are likely to be hydrophobic, since, although a similar change in entropy would be expected from a difference in electrostatic interactions, this would not be consistent with the observed increase in enthalpy (*i.e.* $\Delta\Delta H$ is positive for the formation of the ternary complex from the binary complex). Alternatively, if the first binding event stabilizes a KIX conformation favoring the second binding event, then the entropic barrier to ternary complex formation would be similarly reduced. This latter explanation is supported by the $C\alpha$ secondary shift changes upon MLL binding, which are consistent with a decrease in conformational flexibility in the G_2 helix and C-terminal half of the α_3 helix.

By contrast, the entropy change upon formation of the ternary c-Myb-MLL-KIX complex is unfavorable; the enhancement of c-Myb binding in the presence of MLL is due to a favorable change in enthalpy. Thus, while the magnitude of KIX binding cooperativity is similar for pKID34 and Myb25, the molecular mechanism behind these affinity changes appears to be different in detail. In the case of c-Myb binding,

			*	*	**	
m/hMLL	2736/2840	DCGNILPSDIMDFVLKNTF	2858/2754			
pMLL	3379	DCGNILPSDIMEFVL-NTP	3396			
hMLL2	2104	RPPEDLPSEIVDFVLKNLG	2122			
dCi	1079	VEELILPDEMLQYLNLVKD	1097			
hGli3	1087	NDEDLFPDDVVQYLNSNQ	1105			
m/c/qGli3	1087/1089/1085	NDEDLLPDDVVQYLNSNQ	1108/1107/1105			
xGli3	1085	DDEDLLPDDMVQYLNSRNQ	1103			
hGli2	729	EDDLVLPDDVVQYIKAHAS	746			
xGli2/4	885/890	DDDLMLPDDVVQYIRSNR	903/908			
Consensus		LFPpphhpah				
		123456789				

FIG. 7. Sequence alignment of a group of KIX binding proteins suggests a consensus for allosteric site binding. Shown is the MLL19 peptide sequence encompassing the core region of the minimal activation domain from human (*h*) MLL (24), in alignment with analogous regions from mouse (*m*) and the pufferfish *Takifugu rubripes* (*p*) MLL and the human paralogue MLL2. Similar sequences from regions that were shown to bind KIX are also shown for segment polarity proteins Ci from *Drosophila* (*d*) and human Gli3 (49, 50). Other homologue Gli proteins are from mouse (*m*), quail (*q*), chicken (*c*), and *Xenopus laevis* (*x*). Alignment of activation domains extracted from the NCBI data base were performed using the program BLAST (65). Invariant residues are indicated in *green*, hydrophobic (*h*) in *yellow*, aromatic (*a*) in *magenta*, and acidic or small polar (*p*) residues in *cyan*. Residues that were previously identified to be most important for both KIX binding and transcriptional activation (24) are indicated by *stars*.

cooperativity most likely arises from the formation of stable polar interactions (electrostatic and/or hydrogen bonds) that are present only in the ternary complex and not in either of the binary complexes. However, given the numerous factors that influence the thermodynamics of complex systems, further experiments would be required to firmly establish the molecular basis for cooperativity.

MLL Binds KIX via a Unique Consensus Sequence—Our results demonstrate that the MLL19 peptide encompassing the core region of the transactivation domain (TAD) is capable of high affinity interactions with KIX. Human, mouse, and pufferfish MLL sequences show significant identity in the TAD (Fig. 7), which is unique to this subgroup of trithorax family proteins (46). In addition, a virtually identical copy of the core region of the TAD (MLL residues 2845–2854) is found in a functionally distinct human paralog called MLL2 (47). This same core sequence is crucial for MLL transactivation function, since mutations that were made within the core region led to a reduction of transcriptional activation by MLL, whereas mutations outside it, including deletions N-terminal to Ile-2844, produced no effect (24, 31). Given that a decrease in transactivation potential correlates with impairment of KIX binding activity (24), the core region of the TAD appears to contain all of the necessary elements required for the interaction. Consequently, other KIX-binding proteins with sequences homologous to this core region are also potential candidates for MLL-like interactions.

Surprisingly, sequence homology is low between KIX-interactive domains from different proteins, even for those that have been shown to interact with the hydrophobic pKID-binding groove (*e.g.* CREB and c-Myb). However, we note that there is good agreement between the conserved core of the MLL-TAD and a region from the KIX-binding domain of the *Drosophila cubitus interruptus* gene product Ci (Fig. 7), a transcription factor that mediates segment polarity in early development (reviewed in Ref. 44). Based on sequence similarities between MLL and Ci activation domains, we suggest that Ci might interact with KIX at the MLL-binding site. In support of this mode of interaction, the Ci mutant M1088T is defective for dCBP-mediated transcriptional activation (49), analogous to the disruption of KIX binding caused by the I2849A mutation in hMLL (24). Mammalian homologues of Ci, called glioblast-

toma gene products (Gli), also contain activation domains that are similar to the core of the MLL-TAD sequence (Fig. 7), which for hGli3 was shown to interact with the KIX domain of CBP (50). It is important to note that outside the consensus sequence (Fig. 7), the conserved Leu-2854 of hMLL was shown to be critical for KIX binding (24) but is not conserved in the Gli/Ci sequences, suggesting potential differences between the proposed MLL-like binders.

Although not addressed in our study, it is also possible that sequences not so obviously related to the MLL consensus sequence can bind to the secondary site on KIX. For example, human T-cell leukemia virus type 1 Tax, which mediates transcriptional activation of the viral genome, can interact with the CREB-bound form of KIX (19, 20). Distinct regions of Tax can directly interact with the CREB basic leucine zipper and the CBP KIX domain (51), leading to the proposal that Tax bridges CBP and CREB (19, 20). However, as our studies imply, pKID and Tax interactions with KIX need not be mutually exclusive but instead could be simultaneous and potentially synergistic, with Tax binding at the MLL or other site and pKID interacting at the hydrophobic groove. In support of this hypothesis, while CREB phosphorylation is not required for complex assembly on the Tax-responsive element, it does increase the affinity of KIX for the Tax-CREB-DNA complex (20). Moreover, pKID deletion mutants of CREB disrupt Tax-dependent transactivation from the viral promoter despite demonstrated ternary complex formation (52), suggesting that pKID-KIX interactions may have functional relevance in Tax-mediated transcriptional activation. Similar lines of evidence indicate that KIX may also form a link between p53 and CREB (21, 53) or between Tax and CREB-2/ATF-4 (23).

A Role for KIX in Transcriptional Synergy—In general, assays of transcriptional activation indicate that cooperativity in complex formation can promote transcriptional synergy (54–56). Similarly, an increase in CBP affinity for a particular activator may target a larger proportion of CBP to a specific promoter, leading to enhanced levels of transcriptional activation. Evidence that changes in CBP protein binding affinities can translate into functional differences is provided by a CREB mutant that binds KIX with 5-fold greater affinity and produces a 2-fold increase in CBP-stimulated transcriptional activation of a reporter gene over wild-type levels (57). However, at this point, it remains to be established whether the roughly 2-fold increase in KIX binding affinity for Myb25 or pKID34 caused by MLL19 will translate into comparable increases in the level of transcriptional activation. In addition, it should be noted that further binding cooperativity might be obtained with full-length CREB or c-Myb and MLL via interactions involving other domains, a possibility that remains to be explored.

Whereas it is possible that binding cooperativity itself can augment transcription, additional contributions to activity could result from the recruitment of MLL to a specific promoter sequence. MLL contains a SET domain, which has been shown to bind hSNF5/INI-1 (58), a component of ATP-dependent chromatin-remodeling complexes such as SWI/SNF (55). Although it was proposed that MLL binds to specific promoter sequences via N-terminal AT-hook domains to recruit remodeling complexes, its DNA-binding properties are reminiscent of HMG box proteins that bind nonspecifically to DNA, with a preference for cruciform structures (60, 61). Therefore, any gene-specific function of MLL may require association with other promoter-specific transcription factors. It has been shown that c-Myb activation of certain myeloid genes requires the SWI/SNF complex, which can be recruited by interactions with CCAAT/enhancer-binding protein β , a transcription factor shown to synergize with c-Myb (62). Our results raise the possibility that

CBP interactions with MLL may promote targeting of chromatin remodeling complexes to CREB- or c-Myb-responsive genes to relieve chromatin-mediated transcriptional repression. CBP histone acetyltransferase activity may additionally contribute to this process (1, 59). Interestingly, the histone-binding protein RbAp48 also cooperatively binds to the KIX domain in complex with phosphorylated CREB, leading to an increase in histone binding and acetylation by CBP (22).

Do MLL and c-Myb Act Synergistically in Vivo?—Both MLL and c-Myb are essential for normal blood cell development and appear to have similar roles in this process. In particular, c-Myb is almost exclusively expressed in hematopoietic precursor cells (41, 42), where it is essential for maintenance of the proliferative state; its expression is down-regulated as progenitor cells differentiate into mature blood cells (59). MLL maintains a transcriptionally active state of an array of Hox genes, which, like c-Myb, are universally down-regulated during differentiation (60). Mouse knockout models reveal that a deficiency in either c-Myb or MLL leads to decreased levels of blood cells and severe anemia (30, 43). Parallels also exist in the outcome of defects in either MLL or c-Myb expression, which in both cases deregulate hematopoietic processes. Specifically, chromosomal translocations of MLL are frequently associated with infant and treatment-related leukemias (40), whereas aberrant amplification or truncation of c-Myb has been observed in various types of leukemias (63). Overall, the resemblance between c-Myb and MLL function in both normal and disease states of hematopoiesis, combined with our demonstration that these proteins cooperatively bind the CBP KIX domain, suggest that these proteins could work in concert to regulate some common genes. Considering the correlation between the trans-activation potential of Myb mutants and their ability to transform hematopoietic cells (63), MLL may also increase the leukemogenic potential of Myb, a possibility that remains to be further explored.

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