Published in final edited form as:

J Mol Biol. 2007 September 28; 372(4): 958–969.

MOLECULAR CHARACTERIZATION OF HTLV-1 TAX INTERACTION WITH THE KIX DOMAIN OF CBP/p300

Julita A. Ramírez and Jennifer K. Nyborg*

Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523-1870

Summary

The viral oncoprotein Tax mediates transcriptional activation of human T-cell leukemia virus type 1 (HTLV-1). Both Tax and the cellular transcription factor CREB bind to viral cyclic AMP response elements (vCREs) located in the viral promoter. Tax and serine 133 phosphorylated CREB (pCREB) bound to the HTLV-1 promoter facilitate viral transcription via the recruitment of the large cellular coactivators CBP/p300. While the interaction between the phosphorylated kinase inducible domain (pKID) of pCREB and the KIX domain of CBP/p300 has been well-characterized, the molecular interactions between KIX, full-length Tax, and pCREB have not been examined. In this study we biochemically characterized the interaction between Tax and KIX in a physiologically relevant complex containing pCREB and vCRE DNA. Our data show that Tax and pCREB simultaneously and independently bind two distinct surfaces on the KIX domain: Tax binds KIX at the previously-characterized mixed-lineage leukemia (MLL) protein interaction surface while pCREB binds KIX at the pKID-KIX interface. These results provide evidence for a model in which Tax and pCREB bind distinct surfaces of KIX for effective CBP/p300 recruitment to the HTLV-1 promoter. We also show that MLL competes with Tax for KIX binding, suggesting a novel mechanism of Tax oncogenesis in which normal MLL function is disrupted by Tax.

Keywords

Tax; MLL; KIX; pCREB; HTLV-1

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus responsible for development of a highly aggressive and fatal malignancy known as adult T-cell leukemia (ATL).¹ The HTLV-1-encoded Tax protein causes cellular transformation and is required for strong transcriptional activation of the provirus. Tax stimulates HTLV-1 transcription through interaction with three conserved 21-base pair enhancer elements known as viral cyclic AMP response elements (vCREs) located within the HTLV-1 promoter. The vCRE sequences bind Tax in complex with the cellular transcription factor CREB. Tax binds to minor groove GC-rich sequences flanking the core octanucleotide CRE bound by CREB and interacts with CREB via protein-protein interactions.^{2; 3; 4; 5} Both CREB and Tax associate with the vCRE element as dimers.^{6; 7} Stimulation of many different kinase pathways results in CREB phosphorylation

*Corresponding author: (970) 491-0420 (office); (970) 491-0494 (fax). Email: jnyborg@lamar.colostate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

at serine 133.⁸ Together, Tax and phosphorylated CREB (pCREB) bound to the vCRE elements serve as a high affinity binding site which recruits the large cellular coactivators CBP/p300 to the HTLV-1 promoter.^{9; 10} The quaternary complex consisting of CBP/p300, DNA-bound Tax, and pCREB is believed to strongly activate viral transcription. The KIX domain of CBP/p300 is crucial for Tax-mediated coactivator recruitment. Tax has been shown to interact with three of four major transcription factor interaction domains located within CBP/p300, though the interaction between Tax and KIX is the only one that has been demonstrated to occur when Tax is assembled into the pCREB/Tax/vCRE DNA ternary complex.^{9; 10; 11; 12; 13; 14} The fact that DNA-bound Tax and pCREB simultaneously form a complex with KIX suggests that each transcription factor makes independent contacts with KIX.

The KIX domain of CBP/p300 is composed of three α -helices that form a compact hydrophobic core.¹⁵ It has two well-characterized transcription factor binding surfaces. One surface has been shown to bind pCREB and c-Myb^{15; 16} and the other has been shown to bind MLL and c-Jun binding interfaces.^{17; 18} The phosphorylated kinase-inducible domain (pKID) of CREB, a region that includes the serine 133 phosphorylation site, binds KIX in a hydrophobic groove defined by KIX helices $\alpha 1$ and $\alpha 3$. The transactivation domain of the c-Myb transcription factor binds this hydrophobic groove in a similar fashion as pKID, though the two sequences share no clear similarity.¹⁶ The structure of a ternary complex containing KIX, a domain of the c-Myb transcription factor, and the minimal activation domain of mixed-lineage leukemia (MLL) protein has been determined supporting a two-site model for transcription factor binding to KIX.¹⁷ De Guzman *et al.* found that MLL binds a distinctly separate surface of KIX than the c-Myb/pKID binding surface.¹⁷ These observations suggest the pCREB/Tax/KIX ternary complex may be analogous to the c-Myb/MLL/KIX ternary complex. Another transcription factor, c-Jun, has been shown to occupy the MLL binding site on KIX.¹⁹ Tax was found to compete with c-Jun for KIX binding,¹⁸ providing further support for a two-site binding model in which Tax and pCREB simultaneously bind separate surfaces of KIX. c-Myb and MLL have also been shown to assemble cooperatively with KIX,²⁰ while similarly, KIX binds Tax most effectively when it is assembled into a complex with full-length pCREB and viral CRE DNA.²¹ While both the pKID domain of CREB and a small N-terminal Tax peptide²² have been shown to interact with separate surfaces on KIX, the full-length proteins have not previously been used to characterize these binding sites, nor have they been examined together.

In this report we sought to better characterize the interaction of Tax and KIX in the context of the physiologically relevant quaternary complex that also contains pCREB and vCRE DNA. We show that full-length pCREB and Tax bind KIX at two separate sites. MLL competition with Tax for KIX binding demonstrates Tax and MLL share the same interaction surface of KIX. In addition, KIX constructs carrying mutations in one of the two transcription factor binding sites have enabled us to distinguish independent Tax and pCREB binding to KIX. These findings support a model in which KIX binds the ternary complex composed of full-length Tax, pCREB, and vCRE DNA through simultaneous interaction with both Tax and pCREB on separate interfaces. Our observations also suggest a novel mechanism for Tax oncogenesis through disruption of MLL function.

Results

Tax and pCREB simultaneously bind the KIX domain of CBP

Our first objective was to determine whether full-length pCREB and Tax bind KIX both simultaneously and independently. We reasoned that if Tax and pCREB simultaneously bind to two sites on KIX, saturated binding of one protein to KIX would have no effect on the binding of the second protein. Purified GST-KIX (CBP aa 588–683) was bound to glutathione-agarose beads and used in a GST pull-down assay. Increasing amounts of purified Tax were added to

binding reactions until saturation of GST-KIX was achieved (Figure 1A). We performed several experiments to establish that this concentration of Tax saturated GST-KIX (data not shown). pCREB was then titrated into reactions containing the highest concentration of Tax (Figure 1A). pCREB bound to GST-KIX without displacing Tax. The converse experiment was then performed in which immobilized GST-KIX was incubated with increasing amounts of pCREB until saturation was achieved (Figure 1B). As before, several experiments were performed to determine the concentration of pCREB that saturated GST-KIX (data not shown). vCRE DNA was also included in these binding reactions as we were interested in examining the binding of Tax to GST-KIX in the context of the more physiologically-relevant ternary complex (pCREB/Tax/vCRE DNA) (Figure 1C). For simplicity, figure 1C depicts one KIX molecule binding to the ternary complex though it is unknown whether one or two KIX molecules bind the complex. Tax was then titrated into reactions containing the highest concentration of pCREB (Figure 1B). Tax bound to GST-KIX with no effect on pCREB binding. It should be noted that approximately 40-fold lower concentrations of Tax were used to achieve GST-KIX binding in Figure 1B than in Figure 1A, reflecting the relatively low affinity of free Tax for KIX. The presence of vCRE DNA and pCREB dramatically increases Tax affinity for GST-KIX.²¹ These experiments show that full-length Tax and pCREB each bind KIX independently and can occupy their respective sites simultaneously, supporting a two-site model for pCREB and Tax binding to KIX.

Since we use GST-KIX throughout this study, we performed an electrophoretic mobility shift assay (EMSA) as a control to compare untagged KIX vs. GST-KIX binding to the pCREB/Tax/vCRE DNA ternary complex. Radiolabeled vCRE DNA was incubated with purified pCREB, Tax, and GST-KIX_{588–683} or untagged KIX_{586–680} to form quaternary complexes (Figure 1D, lanes 3–4). The addition of both untagged KIX and GST-KIX to ternary complexes produced an easily observable alteration in mobility, indicating their binding to the complexes. GST-KIX binding resulted in a more slowly migrating quaternary complex than untagged KIX, reflecting the size difference between the proteins and the shape of the resulting complexes. Notably, untagged KIX migrates faster than the ternary complex, likely due to its more compact structure. Comparable amounts of both proteins produced a complete shift in the mobility of the ternary complex (~50 nM, data not shown). As further support for this result, we performed gel filtration chromatography on complexes assembled with either GST-KIX or untagged KIX using a Superdex 200 HR 10/30 column, and found the peak elution volume of each quaternary complex was exactly the same ($V_E = 10.24$ mL, data not shown). The components of each peak were verified using SDS-PAGE.

A small activation domain of Mixed-Lineage Leukemia protein (MLL) competes with Tax for KIX binding

We were next interested in determining whether Tax and the MLL activation domain bind the same site on KIX, as previously hypothesized.¹⁷ A small synthetic peptide corresponding to human MLL (aa 2842–2858) was used for these experiments. This sequence was selected based on previous studies.^{17; 20} The peptide was synthesized with a C-terminal FLAG tag to facilitate concentration determination by absorbance spectroscopy and to provide a convenient negative control for experiments. We first determined whether our MLL peptide actively bound KIX using a fluorescence polarization assay. An N-terminal fluorescein-tagged MLL peptide was incubated with various concentrations of purified untagged KIX_{586–680} peptide and the fluorescence polarization signal was measured (Figure 2A). The K_d for MLL binding to KIX was 2.5 ± 0.3 μ M, almost identical to the previously published value determined by isothermal titration calorimetry.²⁰ We next tested whether MLL peptide bound to KIX could prevent Tax binding, and thus formation of the quaternary complex. Purified GST-KIX_{588–683} was bound to glutathione-agarose beads as in previous GST pull-down experiments and MLL peptide was titrated into the binding reactions. FLAG peptide was added as a negative control. Following

GST-KIX/MLL binding, full-length purified pCREB, Tax, and vCRE DNA were added to the binding reactions. The presence of MLL reduced the amount of Tax bound to GST-KIX while pCREB binding remained unaffected (Figure 2B). The addition of FLAG peptide corresponding to the highest concentration of MLL had no effect on either Tax or pCREB binding, indicating MLL binding to KIX specifically inhibited the binding of Tax, but not pCREB, to GST-KIX.

As an alternative method to examine whether MLL and Tax share the same binding site on GST-KIX, an EMSA was performed. Tax does not stably bind vCRE DNA by itself, therefore, the CREB basic leucine zipper (bZIP) domain was used together with Tax in these assays. We chose to use the bZIP domain to avoid any possibility of an indirect Tax association with KIX via protein-protein interaction with pCREB. Use of the bZIP domain also allowed us to examine the Tax-KIX interaction in the absence of the pKID/KIX interaction. Radiolabeled vCRE DNA was incubated with purified CREB bZIP, GST-KIX_{588–683}, and Tax forming a quaternary complex (Figure 2C, lane 3). Formation of this more slowly migrating complex is dependent upon the binding of KIX to the ternary complex formed with bZIP, Tax, and vCRE DNA. The MLL peptide was titrated into the binding reactions. Figure 2C shows that the MLL activation domain disrupted the interaction between Tax and KIX. To rule out the possibility that the MLL peptide might be interfering with formation of the ternary complex composed of Tax, pCREB, and vCRE DNA, another EMSA was performed in which the MLL peptide was titrated into reactions containing full-length pCREB, Tax, and vCRE DNA. We used full-length pCREB in this experiment as Tax does not bind the bZIP/vCRE DNA complex strongly enough in the absence of KIX to form a detectable ternary complex. The MLL peptide did not interfere with formation of the Tax/pCREB/vCRE ternary complex (Figure 2D). These results strongly suggest that Tax and MLL bind to the same site on the KIX domain.

A carboxy-terminal truncation of KIX demonstrates significantly reduced Tax binding

The C-terminal half of the KIX α_3 helix is extended from E665 to R669 upon MLL binding, creating additional contacts between MLL and KIX (residues 612, 664, 667, and 668).¹⁷ An earlier study found this region of the KIX domain was important for Tax binding, as a truncated KIX mutant (GST-KIX short, CBP aa 588–665) was defective for Tax but not pCREB binding.¹³ We were interested in investigating whether this mutant is also defective for Tax binding in the context of the quaternary complex. Purified wild-type GST-KIX_{588–683} (GST-KIX *wt*) and the truncated GST-KIX_{588–665} (GST-KIX short) proteins are shown in Figure 3A. A GST pull-down assay was first performed to simultaneously test Tax and pCREB binding to GST-KIX short in the presence of vCRE DNA. Tax was titrated into reactions containing GST-KIX *wt* or GST-KIX short and a constant amount of pCREB and vCRE DNA. GST-KIX short showed dramatically reduced Tax binding while pCREB binding remained intact (Figure 3B). We next performed a DNA pull-down assay to test the ability of GST-KIX short to stabilize Tax binding in the pCREB/vCRE complex. For this assay we used a biotinylated vCRE double-stranded oligonucleotide bound to streptavidin-agarose beads. A biotinylated DNA fragment carrying a weak CREB binding site was used as a negative control for Tax binding. The presence of GST-KIX short in the binding reaction did not stabilize Tax on the immobilized template as effectively as wild-type GST-KIX (Figure 3C), demonstrating the importance of the C-terminal half of the KIX α_3 helix for Tax binding in the context of the vCRE-containing quaternary complex.

Mutation of KIX amino acids important for MLL interaction reduces Tax binding

In light of our findings that the MLL peptide competes with Tax for KIX binding and KIX α_3 helix residues are important for Tax as well as MLL binding, we designed a KIX triple point mutant based on conserved, solvent-exposed KIX residues found to interact strongly with MLL (aa F612, D622, and R624).²⁰ We hypothesized that this mutant would be defective for Tax

binding. Using site-directed mutagenesis, we constructed GST-KIX588–683 Δ T (Tax binding site mutant) with these three residues mutated to alanine. The KIX residues targeted for mutation are shown in blue in Figure 4A. Purified GST-KIX Δ T is shown in Figure 4B. A GST pull-down assay was performed using GST-KIX *wt* or GST-KIX Δ T bound to glutathione-agarose beads. Tax was titrated into reactions containing a constant amount of pCREB and vCRE DNA (Figure 4C). Tax binding to GST-KIX Δ T was reduced by approximately two-thirds relative to GST-KIX *wt*, while pCREB binding remained unaffected. These data provide further support for the hypothesis that full-length Tax and MLL share the same binding site on KIX. We also performed a DNA pull-down assay using biotinylated vCRE DNA bound to streptavidin-agarose beads. A biotinylated DNA fragment carrying a weak CREB binding site was used as a negative control for Tax binding. As in Figure 4C, Tax was titrated into reactions containing either GST-KIX *wt* or GST-KIX Δ T in the presence of a constant amount of pCREB (Figure 4D). Tax binding at the vCRE was significantly reduced in the presence of GST-KIX Δ T relative to GST-KIX *wt* (lanes 6–8 vs. 3–5). This experiment demonstrates that mutation of the putative Tax binding site on KIX significantly reduced Tax association with the DNA-bound complex.

A KIX mutant defective for pCREB binding demonstrates wild-type Tax binding

We constructed another KIX mutant, GST-KIX Δ pC, which carries a single point mutation (Y658A) that has previously been shown to be defective for pCREB binding (Figure 5A, purified protein shown in Figure 5B).¹⁵ We reasoned that if a KIX mutant defective for Tax binding can bind pCREB, then similarly, a KIX mutant defective for pCREB binding should be able to bind Tax. To test this theory, a GST pull-down experiment was first performed to confirm that our KIX mutant was indeed defective for pCREB binding. pCREB was titrated into reactions containing either glutathione-bound GST-KIX *wt* or GST-KIX Δ pC (Figure 5C). As expected, we did not detect pCREB binding to GST-KIX Δ pC. The lower panel shows comparable amounts of GST-KIX *wt* and Δ pC were used in the experiment. We next performed another GST pull-down assay to test the ability of GST-KIX Δ pC to bind full-length Tax. Tax was titrated into reactions containing either GST-KIX *wt* or GST-KIX Δ pC (Figure 5D). Tax binding to GST-KIX Δ pC and GST-KIX *wt* was comparable, indicating mutation of the pCREB binding site on KIX does not affect Tax binding.

EMSA confirms Tax binds KIX at an independent site from pCREB

Although similar results were obtained from two different types of immobilization experiments, we confirmed our Tax binding results for the GST-KIX Δ T and Δ pC mutants using an additional method. EMSAs were performed with purified CREB bZIP, Tax, and the various GST-KIX proteins. As in Figure 2C, the bZIP domain of CREB was used in lieu of full-length pCREB to separate the Tax-KIX interaction from the pCREB-KIX and pCREB-Tax interactions. GST-KIX Δ pC and GST-KIX *wt* formed nearly equivalent amounts of the quaternary complex (KIX/Tax/bZIP/vCRE DNA) (Figure 6). However, the GST-KIX Δ T triple point mutant reduced the amount of quaternary complex to approximately one-third that of GST-KIX *wt*. As previously shown,¹³ the truncated GST-KIX short construct did not support detectable quaternary complex formation.

Discussion

The KIX domain of CBP has two separate hydrophobic grooves on opposite sides of the molecule that serve as transcription factor binding sites. Previous studies have demonstrated that one site binds the pKID region of CREB in addition to the activation domain of c-Myb, and the second site binds the activation domain of MLL and a domain of c-Jun.^{16; 17; 19; 20; 23} The HTLV-1 Tax protein, in complex with pCREB and vCRE DNA, serves as a high-affinity binding site for the KIX domain.^{9; 10} The observation that this complex binds KIX

with considerably higher affinity than either protein alone suggests that pCREB and Tax may simultaneously occupy separate sites on KIX. However, full-length Tax and pCREB are much larger than the small domains examined in structural studies of cooperative transcription factor binding to KIX, and steric hindrance could prevent both from simultaneously binding the small KIX domain. In this study, we investigated the molecular interactions between KIX and full-length Tax and pCREB. We also characterized the interaction of the Tax/pCREB/vCRE ternary complex with KIX. As predicted, we found that both Tax and pCREB independently and simultaneously bind KIX. In addition, the MLL activation domain competed with Tax for KIX binding. The C-terminal half of KIX is elongated upon MLL binding, creating additional contacts between MLL and KIX. Our findings suggest Tax interacts with the MLL binding site on KIX in a similar fashion, since a truncated KIX mutant (GST-KIX short) was found defective for Tax binding. In addition, a KIX construct (GST-KIX Δ T) with mutations in the MLL binding site bound Tax with reduced affinity while retaining wild-type pCREB binding. In both cases, mutations in the MLL binding site on KIX disrupted Tax binding, both with Tax free in solution and as part of the transcriptionally-relevant ternary complex. While pCREB and Tax bind KIX in very close proximity, these findings support a model for CBP/p300 recruitment to the DNA-bound Tax/pCREB complex in which Tax and pCREB simultaneously interact with separate binding sites on KIX.

A recent study found that the MLL activation domain transitions from an unstructured to a more highly structured state upon KIX binding.¹⁷ A similar mechanism may occur during Tax binding to KIX in the context of the Tax-containing ternary complex. We have recently found the affinity of Tax for KIX is dramatically increased in the presence of pCREB and vCRE DNA.²¹ Independent and simultaneous transcription factor binding to KIX provides a mechanism by which promoter-bound Tax and pCREB synergistically recruit CBP/p300 to the HTLV-1 promoter.

MLL and Tax competition for the same binding site on KIX raises the possibility that these two transcription factors compete *in vivo* for KIX binding and subsequent promoter recruitment of CBP/p300. This carries implications for an additional oncogenic function of Tax. MLL is a large *Drosophila* trithorax homologue that was originally identified as a gene frequently rearranged in therapy-induced acute myeloid leukemias and childhood leukemias. It positively regulates *Hox* genes and opposes the repressive effect of proteins such as *Bmi-1*.^{24; 25; 26} Tax may disrupt normal MLL function by competition for CBP/p300 binding in HTLV-1 infected cells. Although MLL is usually not rearranged in adult T-cell leukemia patients unless they develop secondary therapy-related myelodysplasia or leukemias,²⁷ Tax expression may mimic the effects of MLL rearrangement in HTLV-1 infected cells by preventing it from activating its target genes. Evidence for Tax-MLL competition for KIX binding thus provides a novel theoretical model for Tax oncogenesis involving disruption of MLL function early in the course of leukemogenesis, during high levels of Tax expression in HTLV-1 infected T-cells.

Materials and Methods

Expression and purification of recombinant proteins

Bacterially-expressed CREB A²⁸, Tax-His₆²⁹ and all GST-KIX¹⁰ proteins were purified to >95% homogeneity as previously described.¹⁰ GST-KIX mutant expression vectors were prepared by site-directed mutagenesis (QuikChange II®, Stratagene), and the incorporation of mutations was verified by sequence analysis. CREB A is a naturally occurring splice variant (aa 1–327) where Ser¹¹⁹ corresponds to Ser¹³³ in human CREB B (aa 1–341).³⁰ To avoid confusion, we will use the Ser¹³³ nomenclature throughout this work. CREB and GST-KIX proteins were dialyzed against TM buffer (50 mM Tris pH 7.9, 100 mM KCl, 12.5 mM MgCl₂, 20% (vol/vol) glycerol, 0.025% (vol/vol) Tween-20, and 4 mM DTT), aliquoted and

stored at -70°C . Tax-His₆ was dialyzed against buffer containing 50 mM Tris pH 8.0, 100 mM KCl, 0.5 M imidazole, 5 μM ZnSO₄ 20% (vol/vol) glycerol, and 4 mM DTT. It was also aliquoted and stored at -70°C . CREB was phosphorylated using the catalytic subunit of protein kinase A by incubating 1.6 μM CREB in a reaction containing 3.3 μM ATP, 5 mM MgCl₂, and 55 units of PKA (New England Biolabs) in a 25 mM potassium phosphate buffer, pH 6.6. Successful CREB phosphorylation was confirmed by the absence of γ -³²P-ATP incorporation following cold phosphorylation. The expression vector for untagged KIX (mouse CBP_{586–680}), a gift from Kevin J. Lumb,³¹ was expressed in *E. coli* BL21(DE3)pLysS. Cultures were harvested by centrifugation, and resuspended in 0.015 volumes of 50 mM Tris-HCl pH7.5, 10 mM EDTA pH 7.5, 0.1 mM PMSF, 8 ng/ μl aprotinin, 8 ng/ μl leupeptin, 1 mini EDTA-free protease inhibitor tablet (Roche, cat #1836170), and 2 mM DTT. Cells were sonicated, the lysate cleared by centrifugation, and applied to Q-sepharose. The flow through containing KIX was applied to two consecutive SP-sepharose columns. KIX was purified to $\geq 98\%$ homogeneity. Cultures were harvested by centrifugation and resuspended in 0.015 volumes of 50 mM Tris-HCl pH7.5, 10 mM EDTA pH 7.5, 0.1 mM PMSF, 8 ng/ μl aprotinin, 8 ng/ μl leupeptin, 1 mini EDTA-free protease inhibitor tablet (Roche, cat #1836170), and 2 mM DTT. Cells were sonicated, the lysate cleared by centrifugation, and applied to Q-sepharose. The flow through containing KIX was applied to two consecutive SP-sepharose columns. KIX was purified to $\geq 98\%$ homogeneity.

MLL peptide

A peptide containing the minimal human MLL activation domain (aa 2842–2858) with a C-terminal FLAG tag was synthesized by Global Peptide. An N-terminal fluorescein-tagged version of this peptide was also obtained from Global Peptide for fluorescence polarization experiments.

Oligonucleotides

The top strand sequence of the complementary oligonucleotides used in the experiments described herein are as follows. The CRE core sequence is underlined and bolded. vCRE: 5'-GAAGATCTCTCAGGCGT**TGACGTC**AACCCCTCACAGATCTTC-3'. Our vCRE construct carries the full vCRE sequence with a single base pair change that converts the off-consensus CRE core to a consensus CRE. It binds Tax indistinguishably from the natural vCRE. A DNA fragment containing a weak CREB binding site (underlined and bolded) was used as a negative control for Tax binding: 5'-GGGGATCT**CTCA**AATATTCTTAGGACCTTTCACCAGATCGGC-3'. The oligonucleotides were purchased from Integrated DNA Technologies (IDT). For the DNA pull-down reactions, a biotin group was chemically added to the 5' end of the upper strand oligonucleotide (IDT).

Antibodies

The antibodies used in the Western blots presented herein were as follows: anti-His (H-3 and H-15), anti-CREB (C-21), anti-phospho-Ser¹³³ CREB, and anti-GST (B-14). All were purchased from Santa Cruz Biotechnologies.

GST pull-down assays

GST pull-down experiments were performed using 10 μl of glutathione-agarose beads equilibrated in pull-down buffer (20 mM HEPES [pH 7.9], 12.5 mM MgCl₂, 10 μM ZnSO₄, 100 mM NaCl, 50 mM KCl, 10% [vol/vol] glycerol, 0.05% [vol/vol] Nonidet P-40). The purified GST proteins were incubated with the equilibrated beads for 1 h at 4°C , washed, and incubated with the second protein(s) for 1 h at 4°C . The reactions were washed two times with pull-down buffer. Bovine serum albumin (0.3 μM) was used to block glutathione agarose beads

between GST fusion and experimental protein binding. Bound proteins were resolved by electrophoresis on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose for subsequent Western blot analysis. The final concentrations of protein and DNA in each reaction are given in the figure legend.

Fluorescence polarization assays

Fluorescence polarization measurements were conducted using a black 384 well flat-bottom polystyrene plate in a Perkin Elmer Victor V model 1420 multilabel counter using 480 nm excitation and 535 nm emission band-pass filters. The 50 μ l reactions contained 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM EDTA (pH 7.5), 20% glycerol, 4 mM DTT, 0.1% NP40, 10 nM N-terminal fluorescein-labeled MLL peptide, and purified untagged KIX_{586–680} at various concentrations from 53 nM to 22 μ M. Reactions were assembled at 4° C and allowed to equilibrate for 30 min at 25° C prior to reading the FP signals. The equilibrium dissociation constant was determined by directly fitting the polarization data to a single-site binding curve using Kaleidagraph (Synergy Software). The K_d value obtained was >100-fold higher than the 10 nM MLL concentration and consequently the free and total KIX concentrations were considered equal when fitting the data. This experiment was performed several times with different KIX protein preparations and similar results were obtained.

DNA pull-down assays

DNA pull-down experiments were performed using streptavidin-coated agarose beads (Novagen). Biotinylated double-stranded oligonucleotides containing a single CRE element were bound to streptavidin-agarose beads by incubating 90 min at 25°C according to the manufacturer's directions. The amount of DNA bound was quantified by measuring the A₂₆₀ of the DNA-containing supernatant before and after streptavidin-agarose bead binding. DNA-bound beads were stored in a 100 mM Na₂HPO₄ pH 7.5, 0.02% sodium azide solution and washed with 0.5X TM buffer before use in assays. Purified proteins were added to aliquots of the streptavidin-agarose bead-bound DNA in 0.5X TM buffer with 0.6 ng/ μ L poly(dA)-poly(dT) and 39 nM BSA added as nonspecific competitors, incubated 45 min at 4°C, and washed three times to remove unbound proteins. DNA-bound proteins were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to nitrocellulose for detection by Western blot analysis.

Electrophoretic mobility shift assays (EMSA)

EMSAs were performed by incubation of the indicated amount of purified CREB, CREB bZIP, Tax, GST-KIX (aa588–683), GST-KIX Δ T (D622A, R624A, F612A), GST-KIX Δ p (Y658A), or GST-KIX short (aa 588–665) in 12.5 mM HEPES pH 7.9, 75 mM KCl, 6.25 mM MgCl₂, 10% (vol/vol) glycerol, 5 μ M ZnSO₄, and 0.05% (vol/vol) NP-40 containing 0.2 nM ³²P-end-labeled vCRE probe and 250 ng/mL poly(dA)-poly(dT) in a 20 μ l reaction volume. Binding reactions were incubated on ice for 15 min. and resolved on 5% nondenaturing polyacrylamide gels [49:1 (wt/wt), acrylamide:N,N'-methylenebisacrylamide] in a buffer containing 0.04 M Tris-HCl, 0.306 M glycine (pH 8.5), and 0.1% (vol/vol) Nonidet P-40. Gels were dried and visualized by PhosphorImager (Molecular Dynamics).

Protein structure graphics

Cn3D 4.1 was used to generate graphics depicting mutated residues in KIX Δ T and KIX Δ pC. The NCBI protein databank code used was 1KDX (MMDB #9136).¹⁵

Image processing

The ImageQuant program (Molecular Dynamics) was used to quantify results. Images were processed in Adobe Photoshop, with minor adjustments made to brightness/contrast as needed

(gamma was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint.

Acknowledgements

We thank Olve Peersen for invaluable suggestions and discussion. We also thank Jeanne Mick for contributing the EMSA shown in Figure 1D, Dinaida Lopez for critical discussion and provision of purified Tax protein, Mara Miller for critical reading of the manuscript, and Sarah Horstmann for help with site-directed mutagenesis. This work was supported by a grant from the National Institutes of Health (CA55035, J.K.N.). J.A.R. was supported by a minority supplement (CA55035-S1).

References

1. Matsuoka M. Human T-cell leukemia virus type 1 (HTLV-1) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology* 2005;2:2–27. [PubMed: 15656908]
2. Lenzmeier BA, Baird EE, Dervan PB, Nyborg JK. The tax protein-DNA interaction is essential for HTLV-I transactivation in vitro. *J. Mol. Biol* 1999;291:731–744. [PubMed: 10452885]
3. Lundblad JR, Kwok RP, Laurance ME, Huang MS, Richards JP, Brennan RG, Goodman RH. The human T-cell leukemia virus-1 transcriptional activator Tax enhances cAMP-responsive element-binding protein (CREB) binding activity through interactions with the DNA minor groove. *J. Biol. Chem* 1998;273:19251–19259. [PubMed: 9668114]
4. Lenzmeier BA, Giebler HA, Nyborg JK. Human T-cell leukemia virus type 1 Tax requires direct access to DNA for recruitment of CREB binding protein to the viral promoter. *Mol. Cell. Biol* 1998;18:721–731. [PubMed: 9447968]
5. Kimzey AL, Dynan WS. Specific regions of contact between human T-cell leukemia virus type I Tax protein and DNA identified by photocross-linking. *J. Biol. Chem* 1998;273:13768–13775. [PubMed: 9593719]
6. Tie F, Adya N, Greene WC, Giam CZ. Interaction of the human T-lymphotropic virus type 1 Tax dimer with CREB and the viral 21-base-pair repeat. *J. Virol* 1996;70:8368–8374. [PubMed: 8970957]
7. Jin DY, Jeang KT. HTLV-I Tax self-association in optimal transactivation function. *Nucleic Acids Res* 1997;25:379–388. [PubMed: 9016568]
8. Johannessen M, Delghandi MP, Moens U. What turns CREB on? *Cell Signal* 2004;16:1211–1227. [PubMed: 15337521]
9. Kwok RP, Laurance ME, Lundblad JR, Goldman PS, Shih H, Connor LM, Marriott SJ, Goodman RH. Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP. *Nature* 1996;380:642–646. [PubMed: 8602268]
10. Giebler HA, Loring JE, Van Orden K, Colgin MA, Garrus JE, Escudero KW, Brauweiler A, Nyborg JK. Anchoring of CREB binding protein to the human T-cell leukemia virus type 1 promoter: a molecular mechanism of Tax transactivation. *Mol. Cell. Biol* 1997;17:5156–5164. [PubMed: 9271393]
11. Scoggin KE, Ulloa A, Nyborg JK. The oncoprotein Tax binds the SRC-1-interacting domain of CBP/p300 to mediate transcriptional activation. *Mol. Cell. Biol* 2001;21:5520–5530. [PubMed: 11463834]
12. Harrod R, Tang Y, Nicot C, Lu HS, Vassilev A, Nakatani Y, Giam CZ. An exposed KID-like domain in human T-cell lymphotropic virus type 1 Tax is responsible for the recruitment of coactivators CBP/p300. *Mol. Cell. Biol* 1998;18:5052–5061. [PubMed: 9710589]
13. Yan JP, Garrus JE, Giebler HA, Stargell LA, Nyborg JK. Molecular interactions between the coactivator CBP and the human T-cell leukemia virus Tax protein. *J. Mol. Biol* 1998;281:395–400. [PubMed: 9698555]
14. Lemasson I, Lewis MR, Polakowski N, Hivin P, Cavanagh M-H, Thebault S, Barbeau B, Nyborg JK, Mesnard J-M. Human T-Cell Leukemia Virus Type 1 (HTLV-1) bZIP Protein Interacts with the Cellular Transcription Factor CREB To Inhibit HTLV-1 Transcription. *J. Virol* 2007;81:1543–1553. [PubMed: 17151132]
15. Radhakrishnan I, Perez-Alvarado GC, Parker D, Dyson HJ, Montminy MR, Wright PE. Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions. *Cell* 1997;91:741–752. [PubMed: 9413984]

16. Zor T, De Guzman RN, Dyson HJ, Wright PE. Solution structure of the KIX domain of CBP bound to the transactivation domain of c-Myb. *J Mol Biol* 2004;337:521–534. [PubMed: 15019774]
17. De Guzman RN, Goto NK, Dyson HJ, Wright PE. Structural basis for cooperative transcription factor binding to the CBP coactivator. *J Mol Biol* 2006;355:1005–1013. [PubMed: 16253272]
18. Van Orden K, Yan JP, Ulloa A, Nyborg JK. Binding of the human T-cell leukemia virus Tax protein to the coactivator CBP interferes with CBP-mediated transcriptional control. *Oncogene* 1999;18:3766–3772. [PubMed: 10391685]
19. Campbell KM, Lumb KJ. Structurally distinct modes of recognition of the KIX domain of CBP by Jun and CREB. *Biochemistry* 2002;41:13956–13964. [PubMed: 12437352]
20. Goto NK, Zor T, Martinez-Yamout M, Dyson HJ, Wright PE. Cooperativity in Transcription Factor Binding to the Coactivator CREB-binding Protein (CBP). *J. Biol. Chem* 2002;277:43168–43174. [PubMed: 12205094]
21. Kim YM, Ramirez JA, Mick JE, Giebler HA, Yan JP, Nyborg JK. Molecular characterization of the Tax-containing HTLV-I enhancer complex reveals a prominent role for CREB phosphorylation in Tax transactivation. *J. Biol. Chem.* 2007in press, April 2007
22. Vendel AC, McBryant SJ, Lumb KJ. KIX-mediated assembly of the CBP-CREB-HTLV-1 tax coactivator-activator complex. *Biochemistry* 2003;42:12481–12487. [PubMed: 14580193]
23. Van Orden K, Giebler HA, Lemasson I, Gonzales M, Nyborg JK. Binding of p53 to the KIX domain of CREB binding protein. A potential link to human T-cell leukemia virus, type I-associated leukemogenesis. *J. Biol. Chem* 1999;274:26321–26328. [PubMed: 10473588]
24. Hanson RD, Hess JL, Yu BD, Ernst P, van Lohuizen M, Berns A, van der Lugt NM, Shashikant CS, Ruddle FH, Seto M, Korsmeyer SJ. Mammalian Trithorax and polycomb-group homologues are antagonistic regulators of homeotic development. *Proc Natl Acad Sci U S A* 1999;96:14372–14377. [PubMed: 10588712]
25. Pirrotta V. Polycomb-ing the genome: PcG, trxG, and chromatin silencing. *Cell* 1998;93:333–336. [PubMed: 9590168]
26. Jacobs JJ, van Lohuizen M. Cellular memory of transcriptional states by Polycomb-group proteins. *Semin Cell Dev Biol* 1999;10:227–235. [PubMed: 10441076]
27. Fukuda N, Shinohara K, Ota I, Muraki K, Shimohakamada Y. Therapy-related myelodysplastic syndrome in a case of cutaneous adult T-cell lymphoma. *Int J Hematol* 2002;75:67–71. [PubMed: 11843294]
28. Franklin AA, Kubik MF, Uittenbogaard MN, Brauweiler A, Utaisincharoen P, Matthews MA, Dynan WS, Hoeffler JP, Nyborg JK. Transactivation by the human T-cell leukemia virus Tax protein is mediated through enhanced binding of activating transcription factor-2 (ATF-2) ATF-2 response and cAMP element-binding protein (CREB). *J. Biol. Chem* 1993;268:21225–21231. [PubMed: 8407959]
29. Zhao LJ, Giam CZ. Interaction of the human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator Tax with cellular factors that bind specifically to the 21-base-pair repeats in the HTLV-I enhancer. *Proc. Natl. Acad. Sci. USA* 1991;88:11445–11449. [PubMed: 1763059]
30. Berkowitz LA, Gilman MZ. Two distinct forms of active transcription factor CREB (cAMP response element binding protein). *Proc. Natl. Acad. Sci* 1990;87:5258–5262. [PubMed: 2142528]
31. Mestas SP, Lumb KJ. Electrostatic contribution of phosphorylation to the stability of the CREB-CBP activator-coactivator complex. *Nat Struct Biol* 1999;6:613–614. [PubMed: 10404213]

Figure 1. Tax and pCREB simultaneously bind the KIX domain of CBP

(a) *pCREB binds to KIX without displacing bound Tax.* GST-KIX_{588–683} (100 nM) was bound to glutathione agarose beads and incubated with increasing amounts of full-length purified Tax (0.7, 1.4, 2.1, 2.8 μ M, lanes 3–6) until GST-KIX was saturated with Tax. pCREB was added in increasing amounts (10 and 20 nM, lanes 7–8) to reactions containing the highest amount of Tax. Tax and pCREB input amounts (25%) are shown in lane 1. As a negative control, pCREB and Tax binding to GST was tested (lane 2). Samples were washed and bound proteins were resolved by 10% SDS PAGE and analyzed by Western blot. An anti-His₆ antibody was used first to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel). The line between lanes 2 and 3 denotes

cropping between lanes of the same experiment. (b) *Tax binds to KIX without displacing bound pCREB*. GST-KIX_{588–683} (25 nM) was used in a GST pull-down assay along with a constant amount of vCRE DNA (500 nM). Full-length purified pCREB was added in increasing amounts (2.5, 5, 25, and 50 nM, lanes 3–6) until GST-KIX was saturated with pCREB. Full-length purified Tax was added in increasing amounts (5, 50, and 75 nM, lanes 7–9) to reactions containing vCRE DNA and the highest amount of pCREB. As a negative control, pCREB and Tax binding to GST in the presence of vCRE was tested (lane 2). Tax and pCREB input amounts (10%) are shown in lane 1. A Western blot was first performed using an anti-His₆ antibody to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel). (c) *Schematic representation of the quaternary complex*. A diagram of the quaternary complex containing Tax, pCREB, KIX, and the viral CRE depicts our model of Tax and pCREB binding to separate surfaces of KIX when in complex with DNA. Although only one KIX molecule is shown, it is unknown whether one or two KIX associate with the ternary complex. (d) *Both untagged KIX and GST-KIX bind the ternary complex*. Electrophoretic mobility shift assays were performed with γ -³²P-end labeled vCRE probe (0.15 nM), pCREB (3 nM), Tax (250 nM) and KIX_{586–680} (250 nM) or GST-KIX_{588–683} (250 nM).

Figure 2. A small activation domain of Mixed-Lineage Leukemia protein (MLL) competes with Tax for KIX binding

(a) *Fluorescence polarization shows MLL peptide binds KIX.* The binding affinity of the MLL activation domain for purified untagged KIX₅₈₆₋₆₈₀ peptide was determined by fluorescence polarization using an N-terminal fluorescein-tagged MLL peptide (aa 2842–2858). The equilibrium dissociation constant was determined by directly fitting the polarization data to a single-site binding curve. (b) *MLL peptide competes with Tax but not pCREB for KIX binding.* GST-KIX₅₈₈₋₆₈₃ (25 nM) was bound to glutathione-agarose beads as in previous GST pull-down experiments and FLAG-tagged MLL peptide (aa 2842–2858) was added in increasing amounts (9.5, 19, and 38 μ M, lanes 5–7) to binding reactions. Following GST-KIX/MLL binding, full-length purified pCREB (25 nM), Tax (35 nM), and vCRE DNA (500 nM) were added to the binding reactions. FLAG peptide (38 μ M, lane 8) was tested as a negative control for Tax binding inhibition. pCREB and Tax binding to GST in the presence of vCRE DNA was also tested as a negative control (lane 2). Tax and pCREB input amounts (20%) are shown in lane 1. A Western blot was first performed using an anti-His₆ antibody to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel). A densitometric analysis of pCREB and Tax binding in this blot is shown below. Numbers on the x-axis correspond to lanes shown in the Western blot. The GST control was set to 1. This data is representative of multiple experiments. (c) *MLL peptide inhibits quaternary complex formation.* Electrophoretic mobility shift assays were performed with γ -³²P-end labeled vCRE probe (0.15 nM), bZIP (7.8 nM), Tax (350 nM) and GST-KIX₅₈₈₋₆₈₃ (312 nM). FLAG-tagged MLL peptide was added to reactions in increasing amounts (12, 24, and 47 μ M, lanes 4–6). FLAG peptide (47 μ M) was added to lane 7 as a negative control. Each nucleoprotein complex is indicated. (d) *MLL peptide does not interfere with ternary complex formation.* An EMSA was performed as before with vCRE probe (0.15

nM), pCREB (2.5 nM), and Tax (250 nM). FLAG-tagged MLL peptide was added to reactions in increasing amounts (20 and 40 μ M, lanes 4 and 5). Nucleoprotein complexes are indicated.

Figure 3. A carboxy-terminal truncation of KIX demonstrates significantly reduced Tax binding (a) Purification of GST-KIX wt and GST-KIX short. Purified wild-type GST-KIX_{588–683} (GST-KIX wt) and GST-KIX_{588–665} (GST-KIX short) are shown Coomassie-stained in a 10% SDS polyacrylamide gel as indicated. (b) Tax binding to GST-KIX short is significantly reduced compared to wild-type GST-KIX, while pCREB binding remains unaffected. GST-KIX_{aa588–683} (25 nM, lanes 3–5) or GST-KIX_{aa588–665} (25 nM, lanes 6–8) were used in a GST pull-down assay in the presence of pCREB (5 nM) and vCRE DNA (500 nM). Full-length purified Tax was added in increasing amounts (5 and 10 nM) as indicated. pCREB and Tax input amounts (20% each) are shown in lane 1. A Western blot was performed using a mixture of antibodies against CREB and His₆. (c) Tax binding at the vCRE is significantly reduced in the presence of GST-KIX short relative to wild-type GST-KIX. Increasing amounts of Tax (20 and 40 nM) were added as indicated to binding reactions containing either GST-KIX_{588–683} (5 nM, lanes 3–5) or GST-KIX short (5 nM, lanes 6–8), biotinylated vCRE DNA (5 nM), and pCREB (7.5 nM). A biotinylated DNA fragment with a weak CREB binding site (lane 2) was included as a negative control for Tax. The pCREB input amount (13%) is shown in lane 1. A Western blot was performed using a cocktail of antibodies against CREB and His₆.

Figure 4. Mutation of KIX amino acids important for MLL interaction reduces Tax binding
 (a) Schematic of wild-type KIX residues mutated in GST-KIX ΔT . Two orthogonal views of KIX (in green) and pKID (in fuschia) are shown in complex with each other as determined by solution NMR (Protein Data bank accession code 1kdx).¹⁵ The three KIX residues shown in blue (F612, D622, and R624) strongly interact with MLL.^{17; 20} These amino acids were mutated to alanine for construction of GST-KIX ΔT . (b) Purification of GST-KIX ΔT . Purified wild-type GST-KIX_{588–683} (GST-KIX wt) and GST-KIX ΔT (F612A, D622A, and R624A) are shown Coomassie-stained in a 10% SDS polyacrylamide gel as indicated. (c) GST-KIX ΔT demonstrates significantly reduced Tax binding. GST-KIX wt (25 nM, lanes 3–6) or GST-KIX ΔT (25 nM, lanes 7–10) was used in a GST pull-down assay along with pCREB (5 nM)

and vCRE DNA (500 nM). Full-length purified Tax was added in increasing amounts (5, 10, and 20 nM, lanes 4–6 and 8–10). Tax and pCREB input amounts (50%) are shown in lane 1. As a negative control, pCREB and Tax binding to GST was tested (lane 2). A Western blot was first performed using an anti-His₆ antibody to detect Tax (lower panel). The blot was then incubated with an antibody to detect CREB (upper panel). (d) *Tax binding at the vCRE is significantly reduced in the presence of GST-KIX ΔT relative to wild-type GST-KIX.* Increasing amounts of Tax (5, 10, and 20 nM) were added to binding reactions containing either GST-KIX *wt* (12.5 nM, lanes 3–5) or GST-KIX ΔT (12.5 nM, lanes 6–8), biotinylated vCRE DNA (5 nM), and pCREB (5 nM). A biotinylated DNA fragment carrying a weak CREB binding site (lane 2) was included as a negative control for Tax. pCREB and Tax input amounts (25%) are shown in lane 1. A Western blot was first performed using an anti-His₆ antibody to detect Tax (lower panel). The blot was then incubated with an anti-CREB antibody and both antibodies were imaged (upper panel).

Figure 5. A KIX mutant defective for pCREB binding demonstrates wild-type Tax binding
 (a) Schematic of wild-type KIX residues mutated in GST-KIX Δp . Two orthogonal views of the KIX-pKID complex are shown with the KIX Y658 residue (in blue) interacting with pKID phosphorylated S133 (in orange). This KIX residue was mutated to alanine for construction of GST-KIX Δp . (b) Purification of GST-KIX ΔpC . Purified wild-type GST-KIX₅₈₈₋₆₈₃ (GST-KIX wt) and GST-KIX ΔpC (Y658A) are shown Coomassie-stained in a 10% SDS polyacrylamide gel as indicated. (c) GST-KIX ΔpC does not detectably bind pCREB. GST-KIX wt (100 nM, lanes 3–5) or GST-KIX ΔpC (100 nM, lanes 6–8) was used in a GST pull-down assay. Full-length purified pCREB was added in increasing amounts (10, 20, and 40 nM) as indicated. pCREB input (25% lowest concentration) is shown in lane 1. As a negative control,

pCREB binding to GST was tested (lane 2). A Western blot was first performed using an antibody recognizing CREB phospho-Ser¹³³ to detect pCREB (upper panel). The blot was then incubated with an anti-GST antibody to detect GST-KIX (lower panel). (d) *Tax binds GST-KIX ΔpC as well as wild-type GST-KIX*. GST-KIX *wt* (100 nM, lanes 3–5) or GST-KIX ΔpC (100 nM, lanes 6–8) was used in a GST pull-down assay. Full-length purified Tax was added in increasing amounts (0.7, 1.4, and 2.1 μM) as indicated. The pCREB input amount (3% lowest concentration) is shown in lane 1. As a negative control, Tax binding to GST was tested (lane 2). A Western blot was performed using an anti-His₆ antibody to detect Tax. The line between lanes 2 and 3 denotes cropping between lanes.

Figure 6. EMSA confirms Tax binds KIX at an independent site from pCREB

Electrophoretic mobility shift assays were performed with γ - ^{32}P -end labeled vCRE probe (0.15 nM), CREB bZIP (5 nM), and Tax (246 nM). GST-KIX *wt* (0.2 and 0.3 μM), GST-KIX ΔpC (0.2 and 0.3 μM), GST-KIX ΔT (0.2 and 0.6 μM), or GST-KIX short (1 μM) were added to reactions as indicated. Each nucleoprotein complex is indicated.