Molecular Characterization of the Tax-containing HTLV-1 Enhancer Complex Reveals a Prominent Role for CREB Phosphorylation in Tax Transactivation*

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Transcriptional activation of human T-cell leukemia virus type 1 (HTLV-1) is mediated by the viral oncoprotein Tax, which utilizes cellular transcriptional machinery to perform this function. The viral promoter carries three cyclic AMP-response elements (CREs), which are recognized by the cellular transcription factor cAMP-response element-binding protein (CREB). Tax binds to GC-rich sequences that immediately flank the CREs. The coactivator CREB-binding protein (CBP)/p300 binds to this promoter-bound ternary complex, which promotes the initiation of HTLV-1 transcription. CREB phosphorylation at serine 133 facilitates transcription from cellular CREs by recruiting CBP/p300 via its KIX domain. However, it remains controversial whether CREB phosphorylation plays a role in Tax transactivation. In this study, we biochemically characterized the quaternary complex formed by Tax, CREB, KIX, and the viral CRE by examining the individual molecular interactions that contribute to Tax stabilization in the complex. Our data show KIX, Ser133-phosphorylated CREB, and vCRE DNA are all required for stable Tax incorporation into the complex in vitro. Consonant with a fundamental role for CREB phosphorylation in Tax recruitment to the complex, we found that CREB is highly phosphorylated in a panel of HTLV-1-infected human T-cell lines. Significantly, we show that Tax is directly responsible for promoting elevated levels of CREB phosphorylation. Together, these data support a model in which Tax promotes CREB phosphorylation in vivo to ensure availability for Tax transactivation. Because pCREB has been implicated in leukemogenesis, enhancement of CREB phosphorylation by the virus may play a role in the etiology of adult T-cell leukemia.

Infection with human T-cell leukemia virus, type 1 (HTLV-1) can cause a rare and ultimately fatal cancer known as adult T-cell leukemia (ATL). Although the vast majority of individuals infected with the retrovirus remain asymptomatic for life, 2–5% go on to develop this aggressive leukemia (1). Expression of the HTLV-1-encoded Tax protein is strongly linked to the development of ATL. Tax is a potent transcription factor that strongly activates transcription, and thus replication, of the HTLV-1 genome. Tax stimulates viral transcription by binding the minor groove of the GC-rich DNA sequences that flank the CRE elements within three conserved 21-bp enhancers (2–5). These enhancers, called viral cyclic AMP response elements (vCREs), are located within the promoter of the provirus. Tax also interacts with the cellular transcription factor CREB, which binds the off-consensus CRE octanucleotide centered within each vCRE. These interactions enable formation of a ternary complex that is critical for activation of viral transcription by Tax.

The Tax-CREB-vCRE complex recruits the multifunctional cellular coactivator CREB-binding protein (CBP) and its parologue p300 to the HTLV-1 promoter, forming a quaternary complex that enables strong transcriptional activation through the intrinsic properties of these coactivators (6–10). CBP/p300 are necessary for mediating activated transcription by a large number of transcription factors, although they do not directly bind DNA. CBP was originally discovered and named for its interaction with protein kinase A (PKA)-phosphorylated CREB (pCREB) (11). Ser133-phosphorylated CREB binds to the KIX domain of CBP (~amino acids 585–680), which is composed of three α-helices that form a compact hydrophobic core (12). A shallow hydrophobic groove on the surface of the core serves as the binding site for the kinase-inducible domain of phosphorylated CREB. Tax also binds KIX, both free in solution and when assembled in the ternary complex (7–9, 13).

The role of CREB phosphorylation in Tax transactivation remains controversial after over a decade of study. Previous studies have shown that CREB phosphorylation was not necessary for optimal Tax transactivation, whereas other studies have found that PKA stimulation increases Tax transactivation (14–16). This question has been difficult to address in vivo because of the pleiotropic effects of CREB phosphorylation and the wide variety of cell lines studied. A basal level of CREB phosphorylation exists even in unstimulated and serum-starved conditions, and phosphorylation-defective CREB mutants may exert global effects on transcription with obvious ramifications for Tax transactivation. The precise role of CREB phosphorylation in Tax transactivation remains elusive.

In this report, we sought to better characterize the detailed...
molecular interactions that contribute to the formation and stabilization of the Tax-containing quaternary complex. We demonstrate that strong Tax binding to the KIX domain of CBP/p300 requires viral CRE DNA and phosphorylated CREB. Additionally, we find that KIX greatly stabilizes Tax binding to the CREBvCRE-DNA complex. Further, we show that the incorporation of phosphorylated CREB is required for formation of a stable complex containing Tax, KIX, and vCRE DNA. Our data support a concerted mechanism of complex formation in which Tax, pCREB, and the KIX domain of CBP/p300 are all required for optimal binding at the HTLV-1 promoter.

Based on these in vitro results showing the importance of pCREB for Tax stability in the complex, we investigated levels of CREB phosphorylation in HTLV-1-infected cells. We observed higher levels of intracellular pCREB in a panel of HTLV-1-infected versus uninfected T-cell lines. Significantly, we found that Tax expression directly enhanced CREB phosphorylation. These observations suggest that Tax promotes CREB phosphorylation in vivo to ensure sufficient pCREB availability for promoter-bound complex formation and robust Tax transactivation.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Proteins**—Bacterially expressed CREB A (17), CREB S133A, Tax-His<sub>6</sub> (18), and GST-KIX (8) proteins were purified to >95% homogeneity as previously described (8). Full-length His<sub>6</sub>-tagged p300 was expressed from recombinant baculovirus in Sf9 cells and purified as previously described (19). CREB A is a naturally occurring splice variant (amino acids 1–327) where Ser<sup>119</sup> corresponds to Ser<sup>133</sup> in human CREB B (amino acids 1–341) (20). To avoid confusion, we will use the Ser<sup>133</sup> nomenclature throughout this work. The KIX domain of CBP used in this study is 85% identical to the p300 KIX domain. Amino acid differences fall largely outside of the minimal KIX domain, which includes the region of pCREB and Tax interaction. All proteins were dialyzed against TM buffer (50 mM Tris, pH 7.9, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 8.0, 20% (v/v) glycerol, 0.025% (v/v) Tween 20, and 1 mM dithiothreitol), aliquotted, 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 MgCl<sub>2</sub>, and 55 units of PKA (Sigma) in a 25-mM potassium phosphate buffer, pH 6.6. Successful CREB phosphorylation was confirmed by the absence of [γ<sup>32</sup>P]ATP incorporation following cold phosphorylation. To confirm PKA singly phosphorylates CREB at Ser<sup>133</sup>, we performed a kinase assay on wild-type CREB and CREB S133A using recombinant PKA catalytic subunit (Sigma) and [γ<sup>32</sup>P]ATP.

**Oligonucleotides**—The top strand sequence of the complementary oligonucleotides used in the experiments described herein are as follows. CRE core sequences are underlined. Fig. 1, A–C, Fig. 2C, Fig. 3A, and Fig. 3C, cellular CRE: 5′-GATCAT-TCCATGACGTCAATTTG-3′; vCRE: 5′-GATCACGCGTT-GAGCACAACCCC-3′ (promoter proximal 21-bp repeat). Fig. 2, A and B, and Fig. 3B: vCRE: 5′-GAAGATCTCTCAG-GGTTGACGTCAACCCCTCACAGTCGTCG-3′. VCRE 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. Modified half CRE: 5′-GGGATCTTCAAATATCTAGGAC-GTCTTACACAGATCGGC-3′. The oligonucleotides were purchased from Integrated DNA Technologies (IDT). For the DNA pulldown 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-15), anti-CREB (C-21), anti--phospho-Ser<sup>133</sup> CREB, anti-Gal4 (DBD), and anti-GST (B-14). All were purchased from Santa Cruz Biotechnologies. An anti-Tax monoclonal antibody (National Institutes of Health AIDS Research and Reference Reagent Program) was also used for detecting transfected Tax.

**GST Pulldown Assays**—GST pulldown experiments were performed as previously described (21). The final concentrations of protein and DNA in each reaction are given in the figure legends. Bound proteins were resolved by electrophoresis on 10 or 12% SDS-polyacrylamide gels and transferred to nitrocellulose for subsequent Western blot analysis.

**DNA Pulldown Assays**—DNA pulldown 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 A<sub>260</sub> of the DNA-containing supernatant before and after streptavidin-agarose bead binding. DNA-bound beads were stored in a 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.5, 0.02% sodium azide solution and washed with 0.5× TTM buffer before use in assays. Purified proteins were added to aliquots of the streptavidin-agarose bead-bound DNA in 0.5× TTM buffer with 0.6 ng/μl poly(dA)poly(dT) and 39 ng bovine serum albumin 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 or a 10–20% gradient SDS gel and transferred to nitrocellulose for detection by Western blot analysis.

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSA were performed by incubation of the indicated amount of purified CREB, Tax, or GST-KIX (588–683) in 12.5 mM HEPES, pH 7.9, 75 mM KCl, 6.25 mM MgCl<sub>2</sub>, 10% (v/v) glycerol, 5 μM ZnSO<sub>4</sub>, 0.05% (v/v) Nonidet P-40, and 0.5 mM EDTA containing <sup>32</sup>P-end-labeled viral CRE probe and 250 ng/ml poly(dA)poly(dT) in a 20-μl reaction volume. Binding reactions were incubated on ice for 30 min and resolved on 5% nondenaturing polyacrylamide gels (49:1 (w/v), acrylamide: Bis) and visualized by PhosphorImager (GE Healthcare).

**Cell Culture and Transient Transfection Assay**—Both HTLV-1-infected (SLB-1, MT-2, C8166) and uninfected (Jurkat, CEM, Molt-4) human T-cell lines were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and penicillin-streptomycin. For whole cell extract preparation, cells were serum-starved by cultivation in the presence of 0.5% serum 24 h prior to harvest.
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Where indicated, cells were stimulated with 10 μM forskolin. Cells were lysed and resuspended in SDS sample dyes. Proteins were separated by 10% SDS-PAGE and analyzed by Western blot. For transient transfection assays, cells were transfected with a constant amount of DNA using FuGENE 6 (Roche Applied Science). After 24 h, the cells were serum-starved (0.5% fetal bovine serum) for an additional 24 h. The cells were harvested, lysed, and analyzed by Western blot analysis. Cells were transfected with expression plasmids for Tax (pSG-Tax) (22), Gal4-CREB (Stratagene), and pUC19 as indicated in the experiment.

**Image Processing**—The ImageQuant program (GE Healthcare) was used to quantify results. Images were processed in Adobe Photoshop, with minor adjustments made to brightness/contrast as needed (γ was kept at 1). No bands were obscured or altered. Images were annotated in PowerPoint. All experiments presented in this report were shown to be reproducible in at least three independent trials.

**RESULTS**

*The Viral CRE and pCREB Enhance Tax Binding to the KIX Domain of CBP*—Transcriptional activation of HTLV-1 requires, in part, the formation of a viral CRE-bound complex composed of Tax, CREB, and CBP/p300. However, a careful biochemical characterization of the precise molecular interactions that contribute to complex stability has not been performed. To carry out this study, we first examined whether Tax binding to the KIX domain of CBP is affected by vCRE DNA and/or pCREB. Purified GST-KIX-(588–683) was bound to glutathione-agarose beads and used in a GST pulldown assay together with full-length, purified, recombinant Tax and increasing amounts of purified, recombinant, PKA-phosphorylated human CREB A (pCREB). Binding reactions were performed in the absence or presence of double-stranded oligonucleotides carrying the viral CRE or cellular CRE DNA. The cellular CRE possesses a higher affinity CREB binding site than the vCRE and lacks the GC-rich flanking sequences required for Tax binding. The cellular CRE thus serves as a negative control for complex formation with Tax. The amount of Tax bound to GST-KIX was determined by Western blot analysis. Fig. 1A shows that Tax was poorly recruited to GST-KIX in the absence of DNA (lanes 2–5). The cellular CRE DNA only modestly enhanced Tax binding, consistent with the fact that this sequence lacks the GC-rich flanks (Fig. 1A, lanes 10–13). The addition of viral CRE DNA resulted in a dramatic increase in the amount of Tax associated with GST-KIX (Fig. 1A, lanes 6–9). Importantly, we found that pCREB is also required for Tax recruitment to GST-KIX, as only small amounts of Tax bound to KIX in the absence of pCREB (Fig. 1A, lanes 2, 6, 10). Titration of pCREB in the presence of the vCRE DNA yielded significantly more Tax binding to the complex (Fig. 1A, lanes 6–9). In binding reactions that contained both vCRE DNA and pCREB, we observed up to a 100-fold increase in Tax association with GST-KIX. In the presence of vCRE DNA, Tax and pCREB binding precisely correlated, further underscoring the importance of pCREB in the Tax-KIX interaction (Fig. 1B). Together, these experiments support previous studies demonstrating the importance of the GC-rich flanking sequences in

**FIGURE 1.** The viral CRE and pCREB enhance Tax binding to the KIX domain of CBP. GST pulldown assays are shown in all four panels. A, tax binding to KIX is strongly enhanced by vCRE DNA and pCREB. Tax (25 nM) was incubated with GST-KIX (588–683) (25 nM) in the absence (lanes 2–5) or presence of vCRE (lanes 6–9) DNA or consensus CRE (lanes 10–13) DNA (500 nM). Phosphorylated CREB (pCREB) was added in increasing amounts (2.5, 25, and 250 nM) as indicated. Samples were washed, and bound proteins were resolved by 12% SDS-PAGE and analyzed by Western blot analysis. Tax input (2%) is shown in lane 1. Tax binding was detected using an anti-His antibody. B, Tax and pCREB binding to KIX precisely correlate in the presence of DNA. Tax (45 nM), vCRE DNA (250 nM), and the indicated amount of pCREB were bound to GST-KIX (250 nM) and assayed as described in panel A. As a negative control, Tax binding to GST (250 nM) was also tested (lane 3). Input Tax and pCREB (0.6 pmol each) are shown in lanes 1 and 2, respectively. Western blot was performed using a mixture of antibodies against CREB and Hist. C, pCREB binding to KIX is modestly enhanced by Tax and vCRE DNA. The experiment shown here is the reciprocal of the experiment shown in panel A. pCREB (25 nM) was bound to GST-KIX (588–683) (25 nM) in the absence (lanes 1–4) or presence of vCRE (lanes 5–8) or consensus CRE DNA (lanes 9–12) (500 nM). Tax was added in increasing amounts (25, 63, and 125 nM) as indicated. pCREB input (20%) is shown in lane 13. Samples were washed, and bound proteins were resolved by 12% SDS-PAGE. pCREB was detected by Western blot analysis. D, Tax directly interacts with KIX. Tax (1 μM) was incubated with GST-KIX (588–683) (100 nM) DNA (lane 3) or GST (100 nM) DNA (lane 2) as a negative control. Input Tax (1 pmol) is shown in lane 1. Molecular weight markers are indicated. Tax was detected by Western blot analysis using an anti-His antibody. All experiments shown in this panel were reproducible in at least four independent experiments.
correlates with KIX binding to the vCRE. Increasing amounts of GST-KIX-(588–683) (2.5, 5, 12.5 nM) were added to immobilized vCRE DNA, as indicated. Lanes 5 and 6 are duplicate reactions, except lane 5 lacks the nonspecific competitors present in all other reactions. Samples were washed, and DNA-bound proteins were separated on a 10–20% gradient SDS-polyacrylamide gel and analyzed by Western blot using an anti-His6 antibody to detect Tax (lane 2), compare lane 1 with a negative control for Tax. The blot was then reprobed with CREB antibodies (lanes 2–5) as a loading control. A biotinylated modified half-CRE with no GC-rich flanks (lane 2) was included as a negative control for Tax; it binds with a reduced affinity compared with the half-CRE (data not shown). These data indicate that the KIX domain, and the Full-length coactivators, play a role in the stabilization of Tax in the quaternary complex. We next evaluated the effect of KIX on Tax binding by performing a titration of KIX-(588–683) into DNA pulldown reactions containing Tax, pCREB, and vCRE’.

We found that Tax binding to the vCRE’ DNA in complex with pCREB was strongly dependent on the presence of KIX in the reaction, giving an ~70-fold increase in Tax binding relative to the absence of KIX (Fig. 2B, upper panel). We also performed DNA pulldown assays using complementary oligonucleotides in which a biotin group was added to the 5′-end of the upper strand, enabling immobilization on streptavidin-agarose beads. The binding site, called vCRE’ , carried the full vCRE sequence with a single base pair change to convert the off-consensus CRE to a consensus sequence. We have shown Tax recruitment to vCRE’ and vCRE is identical; however, CREB binds the vCRE’ with a slightly higher affinity than to the natural vCRE.

We first used the DNA pulldown assay to evaluate the ability of KIX to stabilize Tax in the pCREB-DNA complex. We compared GST-KIX-(588–683) with full-length p300 to assess the physiological relevance of our results. We observed a similar enhancement in Tax binding in the presence of KIX and p300 (Fig. 2A, compare lane 2 with lanes 4–6). We obtained similar results using purified, full-length CBP (data not shown). These data indicate that the KIX domain, and the full-length coactivators, play a role in the stabilization of Tax in the quaternary complex. We next evaluated the effect of KIX on Tax binding by performing a titration of KIX-(588–683) into DNA pulldown reactions containing Tax, pCREB, and vCRE’. We found that Tax binding to the vCRE’ DNA in complex with pCREB was strongly dependent on the presence of KIX in the reaction, giving an ~70-fold increase in Tax binding relative to the absence of KIX (Fig. 2B, upper panel). We also performed DNA pulldown assays using complementary oligonucleotides in which a biotin group was added to the 5′-end of the upper strand, enabling immobilization on streptavidin-agarose beads. The binding site, called vCRE’, carried the full vCRE sequence with a single base pair change to convert the off-consensus CRE to a consensus sequence. We have shown Tax recruitment to vCRE’ and vCRE is identical; however, CREB binds the vCRE’ with a slightly higher affinity than to the natural vCRE.

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Panel, lanes 3–6). Tax and KIX binding precisely correlated in a dose-dependent fashion (Fig. 2B, lower panel). A biotinylated half-CRE site was used as a negative control for Tax binding (Fig. 2B, lane 2). EMSAs with 32P-labeled vCRE DNA were then used to more quantitatively assess the effect of CREB phosphorylation in the complex. We compared the apparent affinity of Tax for pCREB-DNA versus pCREB-KIX-DNA (Fig. 2C). The apparent affinity of Tax for each complex was determined by establishing the Tax concentration at the midpoint of the binding transition from the binary to the ternary, and the ternary to quaternary complex. We found that the apparent affinity of Tax for the pCREB-DNA complex was 60-fold higher in the presence of KIX (Fig. 2C). The binding of Tax reveals the presence of KIX in the complex, as it shifts to a higher position on the gel than pCREB-Tax-DNA (8). This result clearly highlights the importance of the KIX domain in stabilization of Tax in the complex.

**CREB Phosphorylation Is Necessary for Stable Tax Binding to Viral CRE DNA**—To further characterize the molecular interactions that contribute to Tax stabilization in the promoter-bound complex, we next examined the effect of unphosphorylated versus phosphorylated CREB. We performed a GST pulldown assay with immobilized GST-KIX, in the presence of vCRE DNA, and compared the ability of CREB versus pCREB to recruit Tax to the quaternary complex. Fig. 3A shows that pCREB was immeasurably more effective at recruiting Tax to GST-KIX in the presence of vCRE DNA (Fig. 3A). We next used a DNA pulldown assay using the vCRE DNA to further explore differences between CREB and pCREB in mediating KIX stabilization of Tax. As before, Tax binding was dramatically increased in the presence of pCREB and GST-KIX (Fig. 3B, lanes 4–6 versus 8–10).

To further establish the role of pCREB in Tax complex formation, we compared KIX binding to ternary complexes formed with Tax and the two forms of CREB in an EMSA. The addition of Tax promoted ternary complex formation with both forms of CREB (Fig. 3C, lanes 2 and 7). When purified GST-KIX-(588–683) was titrated into binding reactions, quaternary complexes were also observed with both CREB and pCREB (Fig. 3C). However, significantly less KIX was required to form the quaternary complex in the presence of pCREB, consistent with previously published data (8). As a control to confirm that the CREB used in these assays was singly phosphorylated at Ser133, we performed an in vitro kinase assay using the catalytic subunit of PKA. Fig. 3D shows that wild-type CREB was phosphorylated whereas CREB with a serine 133 to alanine point mutation was not.

**Tax Enhances CREB Phosphorylation in Vivo**—The compelling in vitro evidence for the importance of pCREB in Tax recruitment and quaternary complex formation led us to investigate CREB phosphorylation in vivo. We reasoned that pCREB levels might be elevated in HTLV-1-infected cells to ensure maximal Tax transactivation. To address this question, we examined CREB phosphorylation levels in a panel of Tax-expressing, HTLV-1-infected (SLB-1, MT-2, C8166) versus uninfected (Jurkat, CEM, Molt-4) T-cells. Whole cell extracts were prepared from these cells following 24 h of serum starvation. Western blot analysis using an anti-Ser133 phospho-CREB-specific antibody showed significantly higher levels of CREB phosphorylation in the HTLV-1-infected cell lines compared with the uninfected cell lines (≈7-fold), while the total amount of CREB remained unchanged across samples (Fig. 4A). To determine whether levels of CREB phosphorylation could be increased further, we treated the panel of cell lines with forskolin, a CAMP agonist, and examined the levels of CREB phospho-

**FIGURE 3. CREB phosphorylation is necessary for stable Tax binding to viral CRE DNA.** A. pCREB strongly enhances Tax binding to KIX. Increasing amounts of CREB or pCREB (2.5, 25, 250 nm) were added to GST pulldown reactions containing Tax (25 nM), vCRE DNA (500 nm), and GST-KIX-(588–683) (25 nm) immobilized on glutathione-agarose. Tax input (10%) is shown in lane 1. B, pCREB is necessary for Tax binding enhancement by KIX. Immobilized vCRE DNA (5 nm) was added to DNA pulldown reactions containing Tax (12.5 nM), pCREB or CREB (12.5 nM), and increasing amounts of GST-KIX-(588–683) (2.5, 5, and 12.5 nM). As before (Fig. 2B), a reaction containing a modified CRE half-site, pCREB, GST-KIX, and Tax is included as a negative control for Tax (lane 2). Samples were washed, and bound proteins were resolved by 12% SDS-PAGE. Consecutive Western blots were performed using antibodies against Tax (anti-His6), CREB, and GST-KIX (anti-GST). Tax input (20%) is shown in lane 1. Molecular weight markers are indicated at left. C, pCREB, Tax, and KIX are all needed for strong quaternary complex formation. Binding reactions for EMSAs contained γ32P-end-labeled viral CRE DNA probe (0.3 nm/reaction) and constant amounts of CREB (3.6 nm) or pCREB (1.8 nm), in the presence or absence of Tax (95 nm). GST-KIX-(588–683) was added to the reactions in increasing amounts (0.9, 7.4, and 36.8 nm, lanes 3–5 and 8–10, or 300 and 700 nm, lanes 12 and 13). Binding reactions were analyzed on a 5% native gel. Note that the CREB-DNA complex migrates with slightly reduced mobility relative to the CREB-DNA complex. D, CREB is phosphorylated by PKA only at serine 133. In vitro phosphorylation of purified recombinant CREB and a CREB point mutant (serine 133 changed to alanine) was performed in the presence of [γ32P]ATP and PKA. Proteins were resolved on a 12% SDS-polyacrylamide gel and analyzed by PhosphorImager. All experiments presented in this figure were shown to be reproducible in at least three independent trials.
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In this study we characterized the detailed molecular interactions that stabilize Tax in the transcription complex containing CREB, the KIX domain of CBP/p300, and vCRE DNA. We examined the role of the individual components in Tax recruitment to the complex. We found that (i) the viral CRE DNA specifically and strongly enhanced Tax binding to KIX, (ii) pCREB specifically and strongly enhanced Tax binding to KIX, (iii) KIX and full-length p300 enhanced Tax binding to the pCREB-vCRE-DNA complex, and (iv) Ser133-phosphorylated CREB is required for efficient Tax recruitment into the quaternary complex. Notably, we found that intracellular CREB is maximally phosphorylated (relative to forskolin treatment) in HTLV-1-infected cell lines and that Tax expression alone is responsible for promoting high levels of pCREB in vivo. These findings reveal that the virus has evolved a mechanism to elevate pCREB levels in the HTLV-1-infected cell, likely as a mechanism to promote strong Tax transactivation.

Our work demonstrates that multiple protein-protein and protein-DNA contacts contribute to the stability of the Tax-containing quaternary complex. This stabilization is likely essential for the robust transactivation characteristics of HTLV-1 gene expression. Our data indicate that Tax is stabilized in the quaternary complex through interactions with pCREB, KIX, and the viral CRE DNA. Although Tax has previously been shown to bind KIX directly (9, 13, 23–25), the experiments shown here reveal that Tax binding to KIX is dramatically enhanced (~100-fold) by pCREB and vCRE DNA. These data support a model in which pCREB and the vCRE serve as molecular scaffolds that assemble Tax into a structure that is highly competent for interaction with KIX, and thus CBP/p300 recruitment to the HTLV-1 promoter.

Our data shed light on the controversial role CREB phosphorylation has played in Tax transactivation for many years. It is widely believed that Tax bypasses the requirement for CREB phosphorylation in the recruitment of CBP/p300, as studies have shown that KIX binds to Tax in complex with unphosphorylated CREB (7, 8). These data suggest that phosphorylation as before. Fig. 4B shows that, as expected, forskolin treatment increased CREB phosphorylation over 5-fold in the panel of uninfected T-cells. Significantly, forskolin had no effect on the levels of CREB phosphorylation in the panel of infected cells. We conclude that CREB is maximally phosphorylated in these HTLV-1-infected cell lines. We next wanted to determine whether the increased CREB phosphorylation we observed in infected cells is the result of Tax expression. Human 293 cells were co-transfected with expression vectors for Tax and Gal4-CREB (Fig. 4C). Gal4-CREB was used to over-express CREB because the effect of Tax on endogenous CREB was difficult to detect due to low transfection efficiency (~30%, data not shown). Ser133 CREB phosphorylation was enhanced in the presence of Tax, strongly suggesting that Tax is responsible for the elevated levels of CREB phosphorylation observed in the panel of HTLV-1-infected T-cells.

These results reveal that Tax expression promotes elevated pCREB levels in the cell, which is then available to facilitate Tax transactivation through the viral CREs. These in vivo data corroborate our in vitro studies and support a critical role for Ser133-phosphorylated CREB in Tax transactivation. Importantly, these results suggest that an HTLV-1-specific mechanism(s) is in place to ensure constitutively high levels of pCREB in the infected T-cell.

DISCUSSION

In this study we characterized the detailed molecular interactions that stabilize Tax in the transcription complex containing CREB, the KIX domain of CBP/p300, and vCRE DNA. We examined the role of the individual components in Tax recruitment to the complex. We found that (i) the viral CRE DNA specifically and strongly enhanced Tax binding to KIX, (ii) pCREB specifically and strongly enhanced Tax binding to KIX, (iii) KIX and full-length p300 enhanced Tax binding to the pCREB-vCRE-DNA complex, and (iv) Ser133-phosphorylated CREB is required for efficient Tax recruitment into the quaternary complex. Notably, we found that intracellular CREB is maximally phosphorylated (relative to forskolin treatment) in HTLV-1-infected cell lines and that Tax expression alone is responsible for promoting high levels of pCREB in vivo. These findings reveal that the virus has evolved a mechanism to elevate pCREB levels in the HTLV-1-infected cell, likely as a mechanism to promote strong Tax transactivation.

Our work demonstrates that multiple protein-protein and protein-DNA contacts contribute to the stability of the Tax-containing quaternary complex. This stabilization is likely essential for the robust transactivation characteristics of HTLV-1 gene expression. Our data indicate that Tax is stabilized in the quaternary complex through interactions with pCREB, KIX, and the viral CRE DNA. Although Tax has previously been shown to bind KIX directly (9, 13, 23–25), the experiments shown here reveal that Tax binding to KIX is dramatically enhanced (~100-fold) by pCREB and vCRE DNA. These data support a model in which pCREB and the vCRE serve as molecular scaffolds that assemble Tax into a structure that is highly competent for interaction with KIX, and thus CBP/p300 recruitment to the HTLV-1 promoter.

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CREB phosphorylation is not required for Tax recruitment of CBP/p300. However, Giebler et al. (8) found that the $K_d$ for KIX binding to the Tax-CREB complex was much higher than for the Tax-pCREB complex (25 versus 1.7 nM, respectively). Furthermore, we show in Fig. 2C that the $K_d$ for Tax binding to the KIX-CREB complex was much higher than for the KIX-pCREB complex (15 versus 0.25 nM, respectively). These data indicate that pCREB is significantly more effective (~15- to 60-fold) at promoting quaternary complex formation with Tax than the unphosphorylated form of the protein.

We also demonstrate that HTLV-1-infected cell lines contain constitutively high levels of phosphorylated CREB and that this appears to be due directly to the expression of Tax. This observation is in agreement with the idea that pCREB is essential to Tax function and suggests that the virus has evolved a mechanism to ensure high levels of CREB phosphorylation to facilitate viral replication.

Consonant with our hypotheses that Tax promotes CREB phosphorylation and utilizes pCREB for transactivation, kinase inhibitors have been shown to block Tax function in vivo. Interestingly, pCREB has recently been shown to be involved in cellular proliferation and implicated in leukemogenesis (26, 27). Our observation that Tax promotes elevated levels of CREB phosphorylation in vivo may have implications for HTLV-1-dependent malignant transformation. Tax has been shown to activate viral and cellular gene expression through both the ATF/CREB and NF-κB signaling pathways. The role of each of these pathways in Tax-mediated cellular transformation has been extensively studied, yet it remains controversial which pathway is required in this process (28–33). Whether Tax enhancement of CREB phosphorylation is mediated through either of these well-characterized pathways, or via a distinct mechanism of kinase activation, is not yet known. Many kinases are responsible for phosphorylation of CREB at Ser133, and thus our results do not necessarily implicate PKA in Tax-mediated enhancement of CREB phosphorylation (34).

In summary, our work shows CREB phosphorylation is necessary for efficient quaternary complex formation in vitro and implicates pCREB as an essential molecule for Tax function in vivo. Our findings that CREB phosphorylation is elevated in HTLV-1-infected T-cells, and that Tax expression directly enhances CREB phosphorylation, provide further evidence for the integral role pCREB may play in Tax transactivation. We propose that HTLV-1 has evolved a mechanism to ensure high levels of CREB phosphorylation to facilitate viral replication. Our data are consistent with a model in which Tax enhancement of CREB phosphorylation is relevant in the etiology of adult T-cell leukemia.

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