Differential Activation of Viral and Cellular Promoters by Human T-cell Lymphotrophic Virus-1 Tax and cAMP-responsive Element Modulator Isoforms

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We have previously proposed that cAMP-responsive element-binding protein (CREB) activity is stimulated by human T-cell lymphotropic virus-1 (HTLV-1) Tax through two mechanisms that are differentially dependent upon CREB phosphorylation. We have tested this model by examining how Tax affects transcriptional activation mediated by the cAMP-responsive element (CRE) modulator (CREM). The CREM proteins are highly homologous to CREB, particularly in their DNA-binding domains and the kinase-inducible domain (KID), a region that interacts with the coactivator CREB-binding protein (CBP) in a phosphorylation-dependent manner. Despite this similarity, most CREM isoforms are transcriptional repressors. CREMα lacks the glutamine-rich domains found in CREB that are essential for transcriptional activation. We show that the normally repressive CREMα activates the HTLV-1 and cellular CREs in the presence of Tax; activation of the viral element is phosphorylation-independent, and activation of the cellular CRE is phosphorylation-dependent. CREMα(C-G) lacks both the KID and the glutamine-rich regions. This isoform activates the HTLV-1 long terminal repeat in a phosphorylation-independent manner, but does not activate the cellular CRE. This study suggests that Tax, interacting with the basic/leucine zipper region of CREM, recruits CBP to the viral promoter. Tax activation of the cellular CRE depends on the KID and its ability to interact with CBP in a phosphorylation-dependent manner.

Human T-cell lymphotropic virus-1 (HTLV-1) is the causative agent in adult T-cell leukemia (1). The HTLV-1 genome encodes several regulatory proteins that are involved in controlling viral gene expression and pathogenesis. One such protein is the 40-kDa transactivator Tax (2). While Tax contains a transcriptional activation domain, the native Tax protein is unable to bind DNA directly (3, 4). Thus, it has been proposed that the transactivating functions of Tax require its interaction with cellular DNA-binding proteins. Indeed, several basic/leucine zipper transcription factors have been shown to interact with the Tax-responsive elements of the HTLV-1 promoter in vitro, including CREB, CREM, and ATF-1 (5–7). Binding of these factors to the Tax-responsive elements, which resemble the cAMP-responsive elements (CREs) of cellular genes, is markedly enhanced by the Tax protein (8–11). Furthermore, studies have shown that the HTLV-1 long terminal repeat (LTR) can be activated by protein kinase A (PKA) and that the level of activation is augmented by Tax (12). However, the precise molecular mechanisms underlying Tax transactivation are not clear.

Recently, we proposed that Tax potentiates transactivation of cellular CRE-containing genes through a direct interaction with the transcriptional coactivator CREB-binding protein (CBP) (8). We additionally showed that the ability of Tax to function in this manner depends upon CREB phosphorylation, as the augmentation is prevented when PKA is omitted and is significantly decreased when the consensus PKA site in the kinase-inducible domain (KID) of CREB is mutated (8). In contrast, Tax activates expression of the HTLV-1 LTR even in the absence of PKA (8). These results suggest that Tax activates cellular and viral CREs through mechanisms that are differentially dependent on phosphorylation.

The structurally related transcription factors CREB, CREM, and ATF-1 are key mediators of cAMP-regulated gene transcription. While the regulatory properties of ATF-1 remain poorly defined, isoforms of CREB and CREM have well characterized functions as activators and repressors of transcription, respectively. The CREB and CREM genes both encode an amino-terminal phosphorylation domain (KID) flanked by glutamine-rich transcriptional activation domains (13). The KID of CREB has been shown to interact with the transcriptional coactivator CBP in a phosphorylation-dependent manner (14–16). Alternative splicing and selective translational initiation of CREB and CREM transcripts generate a family of proteins ranging from full-length transcriptional activators to truncated transcriptional repressors (17). Specifically, CREMα, which lacks the glutamine-rich domains found in activator isoforms such as CREB and CREMβ, has been shown to be a potent repressor of cAMP-activated transcription (18).
absence of PKA. Thus, we would predict that Tax should convert the represor CREMα into an activator of the cellular CRE because the KID and DNA-binding domain of CREMα are very similar to those of CREB. Moreover, CREMα-mediated activation of the HTLV-1 LTR should occur in the absence of PKA, in a manner similar to that reported previously for CREB. The CREMα(C-G) isoform lacks both the glutamine-rich regions and the KID (19). This form is predicted to be incapable of mediating Tax activation of the cellular CRE, but because it contains a CREB-like basic/zipper region, it should still be able to contribute to activation of the viral LTR. By using a combination of in vivo transfection and in vitro fluorescence polarization binding assays, we show that the Tax activation of cellular and viral CRE-containing genes is indeed mediated through different functional domains of the bZIP proteins in a manner that is differentially dependent upon phosphorylation. Our studies suggest that activation of the viral promoter occurs through the recruitment of CBP and requires, in addition to Tax, only the bZIP domain that is conserved among CREB, CREMα, and CREMα(C-G). In contrast, activation of the cellular CRE by Tax occurs only in the presence of PKA and requires isoforms of CREB or CREM that contain the KID. Thus, recruitment of Tax to the cellular CRE depends upon a phosphorylated KID and its ability to bind to the coactivator CBP.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Assays—F9 teratocarcinoma cells were grown on 0.7% gelatin-coated plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Cells were seeded at 3.5 × 10^5 cells/plate 18 h prior to transfection. DNA was prepared by alkaline lysis, purified twice in CsCl gradients, phenol-extracted, and ethanol-precipitated prior to use. F9 cells were transfected by calcium precipitation (20) with 4 μg of RSV-CREB, 2 μg of RSV-luciferase, 0–8 μg of RSV-Tax, 0–8 μg of RSV-CREMα, 5 μg of p(−71) somatostatin-CAT (21), 0–8 μg of CMV-CREMα(C-G), and 2 μg of pU3R-CAT (22), as indicated. CREMα was a gift from P. Sassone-Corsi; CREMα(C-G) was a gift from J. F. Habener. All other plasmids used in this study have been described previously. The total amount of DNA in each transfection was 30 μg, the balance being made up with Rc/RSV. Cells were washed, refed, and grown for 24 h before harvesting. CAT activity was determined as described previously (23), and values were normalized for luciferase activity as a control for transfection efficiency. RSV-luciferase expression was not affected by introduction of CMV-CREMα(C-G).

Protein Expression—Recombinant CREMα protein was generated using a pET15b expression vector (Novagen). BL21(DE3) cells transformed with a plasmid containing the entire coding sequence of CREMα were grown to an A_600 = 0.6 in Luria broth containing 2 g/liter glucose and 100 μg/ml ampicillin. Cultures were induced to express the recombinant protein by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After 3 h, cells were harvested and lysed by two passages through a French press. Bacterial debris was cleared by centrifugation, and the cleared supernatant was heated to 72°C for 10 min. Precipitated proteins were cleared by centrifugation. CREMα was purified from the supernatant over an FPLC HiTrap Q anion-exchange column (Pharmacia Biotech Inc.). The minimal bZIP CDNA was constructed from CREB/SER (24) by engineering a transcription start site and an Ncol cleavage site at residue 282 using site-directed mutagenesis. This construct, in which the cysteine residues in the basic/zipper region have been changed to serine, was subcloned into the pET15b expression vector using the Ncol/BamHI sites. An oligonucleotide encoding a histidine tag extension (MHHHHHHHSSG) and compatible Ncol ends was ligated to the bZIP/SER vector at the Ncol site to aid in purification. BL21(DE3) cells were transformed with the bZIP/SER plasmid as described above, and cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were centrifuged and resuspended in 300 mM potassium phosphate buffer, pH 7.0, containing 300 mM KCl, 5% glycerol, and 0.1% Triton X-100. Extracts were prepared as described above, and the supernatants were heated to 80°C for 10 min. The peptide was purified over a nickel-nitrilotriacetic acid resin (Qiagen Inc.); dialyzed against 25 mM Tris-ClHCl buffer, pH 8.0, containing 1 mM EDTA, and further purified over an FPLC Q-Sepharose anion-exchange column (Pharmacia Biotech Inc.).

RESULTS

CREMα Activates the Cellular CRE in the Presence of Tax and PKA—CREMα, which lacks the glutamine-rich domains present in the activators CREB and CREM (Fig. 1), generally represses CRE-mediated transcription (18, 28). Our model for Tax activation of CRE-containing genes (8) suggested that CREMα should be converted into a PKA-dependent activator by the addition of Tax. We tested this possibility by measuring expression of a somatostatin-CAT reporter gene in F9 teratocarcinoma cells in the presence or absence of PKA and Tax. F9 cells were chosen for these studies because they lack functional levels of CREB and PKA (29). As shown in Fig. 2, Tax activated expression of the reporter in a dose-dependent manner in the presence of CREMα and PKA (lanes 6–10) by 40-fold. More important, this activation was eliminated in the absence of PKA (lanes 1–5) or in the absence of exogenous CREMα (data not shown). These results demonstrate that CREMα becomes an activator of the cellular CRE in the context of the viral transactivator Tax and that this activation is PKA-dependent.

Activation of the HTLV-1 Promoter through CREMα and Tax Is PKA-independent—Our model additionally predicted that CREMα should be able to mediate the Tax activation of the HTLV-1 LTR and that this activation should not require PKA. To test this possibility, F9 cells were cotransfected with Tax and histidine-tagged Tax and CBP fusion proteins was as described (25, 26).

Phosphorylation—CREMα was phosphorylated using the purified catalytic subunit of PKA (a gift from R. A. Maurer). The phosphorylation reaction was carried out in 50 mM MOPS buffer, pH 6.8, containing 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, and 1 mM ATP at 30°C for 30 min. To measure the incorporation of phosphate, [γ-^32P]ATP was added to the reaction at a final concentration of 0.6 mM. The extent of phosphorylation was determined by trichloroacetic acid precipitable counts. For binding studies, excess ATP was removed by extensive dialysis against binding buffer.

Binding Assays—Fluorescence polarization measurements were performed as described (25, 27). Briefly, a 5- fluororescein-labeled 28-base oligonucleotide representing the sense strand of the promoter proximal HTLV-1 Tax-responsive element (5′-TTCCTAGGGCCTTGGAGAGAC-3′) and a 23-base 5′-fluorescein oligonucleotide representing the somatostatin CRE (5′-CCCTGGCGTCCAGTCAGAG-3′) were annealed to antisense oligonucleotides. An Nterminal fragment of CBP (amino acids 1–682) was titrated in 1 ml of reaction buffer (25 mM, Tris pH 7.6, 50 mM NaCl, 1 mM dithiothreitol 0.1% Triton X-100, 5% glycerol, 6 μg of bovine serum albumin, 10 μg of poly(dI-C), and 5 mM MgCl2) in borosilicate glass tubes containing 5 nM fluorescentl HTLV-1 or somatostatin CRE saturated with either 30 nM CREMα (phosphorylated or non-phosphorylated) or 50 nM bZIP peptide and, in selected experiments, 1 μM Tax. The fluorescence anisotropy of each titration point was determined at room temperature using a PanVera Beacon fluorescence polarization system. Four measurements of each binding condition were averaged for each determination. The apparent K_D for CBP binding was determined from a plot of the anisotropy versus CBP concentration. Binding isotherms were fitted to a simple binding model for the interaction of CBP with CREMα or bZIP peptide, including a linear nonspecific binding component, by nonlinear regression. Protein concentrations were determined by measurement of extinction coefficients and by amino acid analysis.

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and increasing amounts of CREMα in the absence of PKA. The results in Fig. 3 indicate that, in the presence of Tax, CREMα activates the HTLV-1 LTR reporter by 11-fold over basal levels (lanes 1–6). Control transfections indicate that this activation does not occur in the presence of exogenous CREMα alone (lanes 7–11). These results show that the glutamine-rich regions (lacking in CREMα) are not essential for Tax activation of either the viral or cellular CREs. In addition, these data confirm the differential requirement for phosphorylation in Tax-mediated activation of cellular and viral promoters.

Activation of gene expression by PKA-phosphorylated CREB has been proposed to involve the transcriptional coactivator CBP (14–16). Moreover, we have proposed that CBP may participate in the PKA-independent activation of the HTLV-1 LTR mediated by Tax and CREB (8). The evidence that somatostatin and HTLV-1 CRE activation by the repressor CREMα is differentially dependent on phosphorylation suggests that CBP might be involved in these processes as well.

**Tax Does Not Alter CBP Binding to Phosphorylated CREMα in the Context of the Somatostatin CRE**—The conservation of the KIDs of CREB and CREMα suggested that both factors might interact with CBP in a PKA-dependent manner. Binding of CBP to CREMα associated with a somatostatin CRE oligonucleotide was shown by fluorescence anisotropy analysis (Fig. 4A). CBP binds to phosphorylated CREMα with an apparent Kd of 500 nM. In the presence of Tax, the affinity of this interaction is only minimally higher (Kd = 430 nM). As expected, the interaction of CBP with CREMα depends on phosphorylation, as there is no binding of CBP to non-phosphorylated CREMα in the presence or absence of Tax (Fig. 4B). These data indicate that the phosphorylation-dependent activation of the somatostatin CRE reporter by Tax in the presence of CREMα is probably not due to a change in the affinity of the coactivator CBP, but rather to the recruitment of Tax to the promoter via CBP.

**Tax Increases the Affinity of CBP for CREMα and Renders This Interaction Phosphorylation-independent in the Context of the HTLV-1 CRE**—Our model suggests that CREMα and CBP allow activation of the HTLV-1 LTR by Tax in a PKA-independent manner. We characterized the assembly of this complex by fluorescence polarization binding assays. In the presence of Tax, CBP has a higher affinity for phosphorylated CREMα bound to the HTLV-1 CRE (Kd = 130 nM) (Fig. 5A) than to the somatostatin CRE (Kd = 430 nM). In addition, Tax facilitates a high affinity interaction between CBP and the non-phosphorylated form of CREMα (Kd = 140 nM) (Fig. 5B, closed triangles). Recruitment of CBP to the HTLV-1 CRE-non-phosphorylated CREMα complex completely depends on Tax, as there is no specific binding of CBP in the absence of Tax (Fig. 5B, open triangles). These data suggest that the PKA-independent activation seen in the context of the HTLV-1 LTR reporter may be due, in part, to recruitment of CBP. The phosphorylation-independent nature of this interaction suggests that it might involve a region of CREMα distinct from the KID.

**Tax Activates CREMα(C-G) in the Context of the Viral, but Not the Cellular, CRE**—To elucidate the mechanism underlying this differential dependence on phosphorylation, we utilized a CREM isoform, CREMα(C-G), which contains neither the KID nor the glutamine-rich domains, but has a bZIP domain that is nearly identical to that of CREB (19). This factor, like CREMα, is normally a transcriptional repressor. Adya et al. (30) have proposed that Tax interacts directly with a region of CREB shared by the CREM proteins that is amino-terminal to the basic region. Because this region is conserved in CREMα(C-G), we predicted that CBP should be recruited to the viral promoter through a direct interaction between Tax and the conserved bZIP domain of this isoform. Conversely, CREMα(C-G) should bind to the somatostatin CRE, but should be unable to recruit CBP, and therefore Tax, to the cellular promoter. As a result, even though it lacks the glutamine-rich domains and the KID, we predicted that CREMα(C-G) would activate the viral CRE, but not the cellular CRE. We utilized an expression vector encoding CREMα(C-G) in functional assays to test this prediction. F9 cells were cotransfected with CREMα(C-G), Tax, and either the somatostatin or the HTLV-1 LTR reporter. As shown in Fig. 6 (left panel, lanes 1–6), CREMα(C-G) activates the HTLV-1 reporter by 25-fold in the presence of Tax. CREMα(C-G) is unable to activate expression of the somatostatin CRE, however, regardless of Tax (right panel, lanes 1–6). These data support a mechanism of activation in which CBP is recruited to the promoter through different domains of the CREB/CREMα factors, depending on the particular promoter context.

**Tax Facilitates High Affinity Binding of CBP to the CREB bZIP Peptide in an Enhancer-dependent Manner**—Our model predicts that a minimal CREB bZIP peptide should allow Tax to facilitate an interaction between CBP and the HTLV-1 CRE, but should not promote CBP binding to the somatostatin CRE.
Indeed, as seen in fluorescence polarization binding assays (Fig. 7, closed circles), CBP binds to the HTLV-1 CRE saturated with bZIP peptide in the presence of Tax ($K_d = 306 \text{ nM}$). There was no specific interaction between CBP and bZIP peptide in the absence of Tax (data not shown). In addition, no specific binding of CBP to the somatostatin CRE saturated with bZIP peptide was detected, regardless of the presence of Tax (Fig. 7, open circles). These data support our model for differential activation of viral and cellular CRE-containing genes. Specifically, CBP is recruited to the Tax-responsive viral CRE through direct interaction between Tax and the basic domain conserved among CREB, CREMα, and CREMΔ(C-G). Recruitment of CBP, and therefore Tax, to the cellular CRE depends on the phosphorylation of the KID conserved in CREB and CREMα.

**DISCUSSION**

The finding that CREB participates in Tax-mediated activation of the HTLV-1 and cellular CREs, along with the high degree of conservation among regions of CREB and CREM, suggested that Tax might also interact with some CREM isoforms. Indeed, several lines of evidence have supported this possibility (31, 32). Whether the interaction of Tax and CREM would result in activation or repression of transcription of viral
and cellular CREs was unclear, however. Many of the CREM isoforms, including CREMα and CREMΔ(C-G), are known to be inhibitors of transcription (18, 19). One model suggests that Tax functions by increasing dimerization of bZIP transcription factors, thereby enhancing binding of these factors to CREs (9, 11). If this were the primary mode of Tax-mediated gene activation, we would expect that Tax would enhance repression by CREMα, rather than cause activation. The fact that CREMα activates both viral and cellular CRE-containing genes in the presence of Tax and that activation of the two types of promoters is differentially dependent on phosphorylation argues for an alternative mode of action for the Tax transactivator.

The CREB transcription factor has provided a good model for understanding the role of phosphorylation in gene activation. We (14, 15) and others (16) have shown that phosphorylation of CREB by PKA facilitates a high affinity interaction between the KID and the coactivator CBP. The KID is highly conserved between CREB and CREMα, suggesting that CBP could interact with the repressor upon phosphorylation by PKA. Here, we show that the binding affinity of CBP for phosphorylated CREMα is equivalent to that of CREB. Although phosphorylated CREMα interacts with the coactivator CBP in vitro, the repression of cellular CRE-containing genes by CREMα in vivo suggests that this interaction is not sufficient for gene activation. The lack of the glutamine-rich domains in CREMα may contribute to this repression by failing to recruit other activators to the CREMα-CBP complex or by failing to provide additional interactions with components of the basal transcriptional machinery. This report shows that phosphorylated CREMα becomes an activator of the somatostatin promoter in the presence of Tax. Presumably, recruitment of the activation domain in the Tax C terminus (33–35) by CBP (8) allows CREMα to participate in activating the cellular promoter. The idea that CBP is an essential component of this activation process is supported by our finding that the activation domain of Tax cannot induce expression from the somatostatin CRE in the presence of CREB or CREM isoforms that are incapable of interacting with the coactivator CBP, i.e. CREB-M1 (8) or CREMΔ(C-G). In contrast, CREM isoforms lacking only the glutamine-rich domains can still participate in Tax-mediated activation of cellular CREs if they are capable of interacting with CBP.

The PKA-independent activation of the HTLV-1 CRE by Tax and CREMα may provide further insights into the mechanism of Tax action. Adya et al. (30) have suggested that a conserved Ala-Ala-Arg motif flanking the CREB basic region is necessary for a direct Tax-CREB interaction. This motif is conserved in CREMα, suggesting that a direct Tax-bZIP interaction may be sufficient for activation of the HTLV-1 CRE. By utilizing another repressor isoform of CREM, CREMΔ(C-G), which contains the conserved bZIP domain but not the KID, we demonstrated that the KID is not essential for PKA-independent viral promoter activation. Binding assays suggest that this activation is due to the ability of Tax, interacting with the minimal bZIP domain, to recruit the coactivator CBP to the viral promoter. Thus, Tax may provide a bridge between the HTLV-1 promoter and the coactivator CBP through a mechanism that does not depend on phosphorylation. The different modes of Tax action emphasize that while Tax cannot interact directly with DNA, the sequence of viral and cellular Tax-responsive elements has a strong influence on the behavior of this transactivator.

The influence of promoter sequence on the behavior of specific transcription factors is well established (36). Indeed, while CREMα is primarily believed to be a repressor of cAMP-mediated transcription, there is some evidence that its ability to
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stimulate or repress gene transcription depends upon the promoter context of the CRE sequence (37). How promoter context influences CREM activity is unclear, however. In this paper, we provide evidence that CREM can activate transcription of viral and cellular promoters in the presence of Tax in a manner that is differentially dependent on phosphorylation. Specifically, we demonstrate that while phosphorylated CREM can interact with the coactivator CBP in a manner similar to CReB, this interaction does not result in activation of a cellular CRE. However, additional recruitment of the viral transactivator Tax through CBP results in a complex that promotes transcription. It remains unclear, however, whether activation domains in CBP are essential for activation of the HTLV-1 CRE or whether the activation domain of Tax functions through other coactivators or components of the basal transcriptional machinery (38).

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