Interaction of the human T-cell lymphotrophic 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

PROTEIN–DNA INTERACTION/TRANS-ACTIVATION/TAX ACTIVATION FACTOR/TAX PURIFICATION

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ABSTRACT The human T-cell lymphotrophic virus type I (HTLV-I) Tax protein activates transcription from three 21-base-pair (bp) repeat sequences in the viral enhancer. Using gel electrophoretic mobility-shift assays, we now show that Tax interacts directly with the nuclear proteins, Tax activation factors (TAFs), that bind the 21-bp repeats. This interaction is demonstrated by decreased electrophoretic mobilities of the TAFs–21-bp-repeats complexes upon supply of Tax exogenously. Formation of the TAFs–21-bp-repeats and Tax–TAFs–21-bp-repeats complexes correlates with in vivo transactivation by Tax. Furthermore, interaction of Tax with TAFs enhances their binding to the 21-bp repeats. These data indicate that transactivation by Tax is most likely mediated by interaction of Tax with TAFs.

Human T-cell lymphotrophic virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia/lymphoma (ATL) (1). Most recently HTLV-I has been associated with a neurological disorder called tropical spastic paraparesis (also known as HTLV-I-associated myelopathy) (2–5). Similar to other viruses such as simian virus 40 (Tag), adenovirus (Ela), herpes simplex virus (VP16), and human immunodeficiency virus (HIV) (Tag), HTLV-I encodes a 40-kDa nuclear protein, Tax, which stimulates transcription from three 21-base-pair (bp) repeat sequences in the viral enhancer (6–14). The key element in the 21-bp repeat responsible for Tax activation is a sequence motif TGACG(T/A) (15–19), which appears in the enhancer/promoter region of numerous cellular and viral genes (20–22). Tax has also been shown to activate expression of interleukin 2, interleukin 2 receptor α-chain genes, and HIV enhancer via the NF-κB binding site (23–31). As Tax does not bind DNA directly, it has been proposed that trans-activation by Tax is mediated by NF-κB-like factors and cellular transcription factors that interact with the 21-bp repeats (15–19). The molecular mechanisms through which Tax affects these transcriptional factors remain unknown largely due to the lack of an in vitro system for dissecting the biochemical events orchestrated by Tax. We now report evidence showing that Tax interacts with the cellular factors that specifically bind the 21-bp repeats.

MATERIALS AND METHODS

Construction of Plasmids Expressing TaxH4. Plasmid pX5 contains the tax open reading frame under control of bacteriophage T5 P21 promoter (32). This plasmid was linearized with HindIII and used as the template for PCR with primers 1 (ATGAGCCCGAAAATCTC) and 2 (TGAGCCATATGG-TATTAGGTGGAGGTGGCTGGACTTCTGTTCTCGGAA) (Fig. 1A). The PCR-amplified DNA was digested with Xma I and ligated to the pX5 plasmid digested with Xma I and EcoRV to generate pTaxH6. The Acc I–BamHI fragment of pTaxH6 was inserted into a mammalian Tax expression plasmid, HTLV-tat I (34) at the Acc I and Bgl II sites to generate pLTR-TaxH6 for transient expression in mammalian cells.

Purification of TaxH4 Fusion Protein. Escherichia coli HB101 cells containing pTaxH4 were grown, harvested, treated with EDTA/lysozyme, and sonicated. The concentrated supernatant was then precipitated with ammonium sulfate at 25% saturation (32). The precipitate was dissolved in buffer A (50 mM sodium phosphate, pH 7.8/0.5 M NaCl/0.5 mM diithiothreitol/0.5 mM phenylmethylsulfon fluoride/5 mM imidazole), loaded on a Pharmacia separating Sepharose 6B column precharged with Ni2+ and eluted with an imidazole gradient of 0–0.4 M in buffer B [50 mM sodium phosphate, pH 7.2/0.3 M NaCl/10% (vol/vol) glycerol]. Fractions containing TaxH4 were dialyzed against buffer C [50 mM Hepes, pH 7.9/50 mM KCl/0.5 mM MgCl2/0.1 mM EDTA/0.5 mM phenylmethylsulfon fluoride/0.25 mM diethiothreitol/20% (vol/vol) glycerol], aliquoted, and frozen in –70°C.

Construction of 21-bp-Repeat Mutants. The sequence of the Xho I–Nco I fragment in plasmid p13 (18) used for gel electrophoretic mobility-shift assays is as follows: 5′ (Xho I)-TGGAGAGGCCTGAGGTCTGCCTCCCCAGATCTCGG-GCTTGGACGACAAACCCCTACCTGAAATGCTTCATG-(Nco I) 3′; TGACG(T/A) motif is in boldface letters. In a derivative of p13, p12, the first thymine of the promoter distal copy of the TGACG(T/A) motif was mutated to guanine. This base substitution abrogates in vitro transactivation of p12 by Tax (18). An identical mutation in the promoter proximal TGACG(T/A) motif of p12 was created by replacing the Bgl II–Nco I fragment with a double-stranded oligonucleotide containing the base substitution to yield plasmid p221.

Gel Electrophoretic Mobility-Shift Assay. Xho I–Nco I fragments (70 bp) containing two copies of wild-type (p13) or mutant (p221) 21-bp repeats were excised from the respective parent plasmids and labeled with 32P by filling in the 3′- recessed ends. DNA-binding reaction was as described (18). After incubation for 30 min at 37°C with or without 0.1 μg of purified TaxH4, the reaction mixtures were chilled on ice and electrophoresed in a 4% polyacrylamide gel in 25 mM Tris, pH 8.5/192 mM glycine/1 mM EDTA buffer at 4°C until the tracking dye xylene cyanol migrated ~12 cm from the origin.

Abbreviations: HTLV-I, human T-cell lymphotrophic virus type I; TAF, Tax activation factor; LTR, long terminal repeat; HIV, human immunodeficiency virus.

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RESULTS

Expression and Rapid Purification of TaxH6. To understand the mechanism of Tax action, we previously constructed a plasmid pX5 that expresses biologically active Tax (32). To facilitate purification of Tax, we introduced six histidine residues at the COOH terminus of Tax by a PCR approach (Fig. 1A). The histidine extension chelates transition metal ions, such as Ni²⁺ or Zn²⁺ via coordination chemistry and allows rapid purification of recombinant proteins by metal ion-chelating Sepharose (34). The TaxH6 recombinant protein expressed from pTaxH6 was purified on a metal-chelating Sepharose column charged with Ni²⁺ (Fig. 1B). TaxH6 eluate peaked at 0.3 M imidazole (lane 15) and remained soluble after dialysis. When the TaxH6 coding sequence was placed under control of the HTLV-I long terminal repeat (LTR), the resultant plasmid, pLTR-TaxH6 (Fig. 1A), trans-activated the appropriate HTLV-I LTR chloramphenicol acetyltransferase construct (6), pU3RCAT, as the wild-type Tax construct, HTLV-tat 1 (34), upon cotransfection into Jurkat cells (Fig. 1C). Hence, TaxH6 is functionally indistinguishable from the wild-type Tax.

Conversion of Specific Tax Activation Factors (TAFs)-21-bp-Repeats Complexes into Lower Mobility Forms by Tax. Protein–protein interaction has been shown to be the mechanism of transcriptional activation of several viral transactivators such as herpes simplex virus VP16 (35, 36), adenovirus E1a (37), and, more recently, hepatitis B virus pX (38); however, to date, it is not known whether analogous mechanisms are used by the HTLV-I Tax in trans-activation. Because at least two copies of the HTLV-I 21-bp repeats are required for optimal Tax action, we carried out gel electrophoretic mobility-shift assays using Jurkat nuclear extract and a 70-bp Xho I–Nco I fragment (18) containing two copies of the 21-bp repeats. Fig. 2A shows that Jurkat nuclear extract yielded mainly three protein–DNA complexes (I–III; lane 1). TaxH6 addition decreased the electrophoretic mobilities (super-shift) of complexes I and II (lane 2), yielding complexes Ia and II, TaxH6 alone without nuclear extracts produced no observable bands (Fig. 3A, lane 4), indicating that TaxH6 has no affinity or low affinity for DNA, as has been reported (18). Complex III was not altered by TaxH6 and served as a useful internal control for monitoring the effect of Tax on complexes I and II. We also noticed protein–DNA complexes with slower mobilities than complexes I–III; their formation was enhanced when Tax was added. We think these complexes are probably other higher-order forms of complexes I–III or of complexes Ia and IIa.

To show that the observed effect of TaxH6 on mobilities of complexes I and II was not from the histidine residues at the COOH terminus, we added to the assay the same amount of a recombinant HIV-1 reverse transcriptase modified to contain six histidine residues at the NH₂ terminus (from Stuart Le Grice, Case Western Reserve University). Fig. 2A lane 3 indicates this HIV-1 reverse transcriptase did not change complexes I–III. Furthermore, wild-type Tax partially purified from E. coli harboring pX5 (18) altered the mobility of complexes I and II similarly as TaxH6 (lane 4). That conversion of complexes I and II to Ia and IIa was due entirely to exogenously added TaxH6 was further shown by the depletion of TaxH6 by Tax antibodies and the subsequent loss of TaxH6 effects on mobility changes of complexes I and II. As shown, treatment of TaxH6 with Tax antibody (Fig. 2B, lane 2) or Tax-C antibody (prepared against the COOH-terminal 33 amino acid residues of Tax, provided by Bryan Cullen,
many cell types including HeLa cells. We, therefore, tested the ability of HeLa nuclear extract for TAFs. Fig. 2C, lane 5, shows that HeLa nuclear extract also gave rise to bands I–IV and several other additional bands. When TaxH6 was added (lane 6), again mobilities of complexes I and II but not others shifted specifically. These data lend further support to the conclusion that complexes I and II are the specific targets of Tax.

In binding reactions containing lower amounts of TaxH6, at least one complex migrating at an intermediate position between bands I and I* appeared (Fig. 2D). With 50 ng of TaxH6, three bands were discernible (lane 4). Higher amounts of TaxH6 shifted complex I to the position of I* (lanes 5 and 6). In our assays, 100 ng of TaxH6 preparation seemed sufficient for complete conversion of complex I to I*.

It is possible that the stoichiometry of Tax may vary as amounts of TaxH6 in the assay alter. We noted that with increased amounts of TaxH6, intensities of bands I* and I** increase, suggesting that Tax enhances binding of TAFs to the 21-bp repeats (see below).

**Formation of Complexes I, II, I*, and I** Correlates with Tax Trans-activation in Vivo.** Previous studies showed that mutations in the HTLV-I 21-bp repeat that abolish trans-activation by Tax in vivo lie primarily in a CRE (cAMP responsive element) -like motif [TGACGT(T/A)] (18). To correlate the formation of complexes I, II, I*, and I** with biological functions, a DNA construct containing point mutations in both copies of the 21-bp repeat was made. Each mutant repeat contains a point mutation that alters the TGACGT motif to GGACGT, a base substitution shown (18) to abolish Tax trans-activation in vivo. Fig. 3A shows that mutations in the TGACGT(T/A) motif completely abolished formation of complexes I, II, and I** in the absence of TaxH6 (lane 5) and complexes I* and I** in the presence of TaxH6 (lane 6). Even in reactions where three times more mutant DNA fragment was used (lanes 7 and 8), no complexes I, II, and IV (lane 7) or I* and I** (lane 8) were seen. These data showed that the *in vitro* formation of complexes I, II, I*, and I** can be correlated with *in vivo* transactivation by Tax. Although complex IV was apparently not affected by Tax, its formation was also abolished by the mutation in the TGACGT(T/A) motif. To further demonstrate specificity of complexes I and II, competition experiments were done. Fig. 3B shows that complexes I, II, IV, I*, and I** were competed against efficiently by increased amounts of unlabeled wild-type HTLV-I 21-bp repeats (lanes 1–8) but not by the mutant repeats (lanes 9–16).

**Binding of Tax to TAFs.** The simplest explanation for the Tax-induced decrease in electrophoretic mobilities of complexes I and II is that Tax is physically bound to TAFs–DNA complexes I and II and converts them to complex I* and I**. To test this possibility, anti-Tax-C IgG-purified from protein A–Sepharose column was directly added to the binding reactions. Fig. 4 lane 4 shows that addition of anti-Tax-C IgG to the reaction containing TaxH6 resulted in the formation of an additional large-molecular-size DNA–protein complex (indicated by triangle). This complex is specific for the anti-Tax-C IgG because β-galactosidase IgG added to the reaction containing TaxH6 did not induce any changes (lane 5). The large-molecular-size species forms only when the binding reaction contains TaxH6. Addition of anti-Tax-C IgG to the reaction not containing TaxH6 had no effect (lane 3). These data indicate that Tax is directly present in complexes I* and I**, and binding of anti-Tax-C IgG converts complexes I* and I** to the large-molecular-size complex(es), causing the decreased intensities of complexes I* and I**. When increased amounts of anti-Tax-C IgG were added to the reactions (lanes 7 and 10), larger complexes formed that remained at the origin of sample application. These complexes were most likely due to oligomerization of the IgG–
Enhanced Binding of TAFs to the 21-bp Repeats by Tax. We repeatedly noticed that when TaxH6 is added to the binding reactions the levels of complexes Ix and IIx significantly increase relative to control (Fig. 2 C and D); the level of complex IV was also increased. To further investigate the relative stability of complexes I and II vs. Ix and IIx, binding reactions were done with increased amounts of competing poly(dI-dC). Fig. 5 shows that high concentrations of poly(dI-dC) more efficiently reduced the levels of complexes I and II than those of Ix and IIx. These results suggest that with Tax the affinity of TAFs for the 21-bp repeats is increased such that TAFs become more resistant to the competing nonspecific DNA. The stabilization of complexes Ix and IIx by Tax has interesting implications for the in vivo trans-activation by Tax where the Tax-responsive cis regulatory sequences constitute only a minuscule portion of the chromosomal DNA.

**DISCUSSION**

In this paper, we demonstrate that HTLV-I Tax directly interacts with the cellular factors, TAFs, that bind the 21-bp repeat elements in the viral enhancer. Tax binding to the TAFs–21-bp-repeats complexes I and II decreased elec-
phonetic mobilities of these complexes. Furthermore, direct interaction of Tax with TARs enhances their binding to the 21-bp repeat element. Mutation and competition experiments showed that complexes I, II, and IV are specific for the TGAGC(T/A) motif in the 21-bp repeat element. Mutations that abolish in vivo trans-activation by Tax also abolished the formation of all three complexes.

A number of cellular factors (ATF, TREB, CREB, CRE-BP1) that bind to the CRE and/or the 21-bp element have been cloned and sequenced recently (41-46). These cellular factors all contain leucine zipper domains at their COOH termini, some of which can engage in heterodimer or homodimer interactions (43). Possibly TARs are among these cloned factors. The assay described in this report should facilitate their identification. Because the interleukin 2, interleukin 2 receptor a-chain genes, and HIV enhancer are also activated by Tax via the NF-xB binding sites, determining whether similar interactions can also occur between Tax and the NF-xB-like factors would be of interest.

In summary, our results provide biochemical evidence that HTLV-I Tax interacts with the cellular factors, TARs, that bind the 21-bp repeats. This interaction alters the TARs-DNA complexes I and II qualitatively and enhances TARs binding to the 21-bp repeats quantitatively. These changes most likely are responsible for the transcripational activation mediated by Tax. An in vitro assay for Tax function described in this study should help reveal the molecular details of Tax action.

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