Cell-Type-Specific Transactivation of the Parathyroid Hormone-Related Protein Gene Promoter by the Human T-Cell Leukemia Virus Type I (HTLV-I) Tax and HTLV-II Tax Proteins

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The human T-cell leukemia virus type I (HTLV-I) and HTLV-II Tax proteins are potent transactivators of viral and cellular gene expression. Using deletion mutants, the downstream parathyroid hormone-related protein (PTHrP) promoter is shown to be responsive to both HTLV-I and HTLV-II Tax as well as the AP1/c-jun proto-oncogene. Transactivation of PTHrP by Tax was seen in T cells but not in B-cell lines or fibroblasts. A carboxy terminal Tax deletion mutant was deficient in transactivation of both the PTHrP and IL2Ra promoters but not the HTLV-I long terminal repeat (LTR). Exogenous provision of NFKb rescued IL2Ra expression but not the PTHrP promoter. Thus, HTLV-I Tax, HTLV-II Tax, and c-jun transactivate PTHrP and may contribute to the pathogenesis of hypercalcemia in adult T-cell leukemia.

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MATERIALS AND METHODS

Materials. All restriction and DNA modifying enzymes were purchased from Bethesda Research Laboratories (Bethesda, MD). [14C] chloramphenicol and [α-32P]dNTP were purchased from Amersham (Arlington Heights, IL).

Cell culture. MLA 144 T cells, 729 B cells, and 729 pheno cells were maintained in Iscove’s modified Dulbecco’s medium with 10% fetal calf serum (FCS). ATLL 1K cells (mutant HTLV-I genotype) were maintained in Iscove’s medium with 20% FCS. MT2 cells were maintained in RPMI 1640 medium with 10% FCS, and Rat 1 cells were grown in α minimum essential medium with 10% FCS. Medium contained 50 U/mL penicillin, 50 μg/mL streptomycin, and 1-glutamine.

Plasmids. A 2,784bp HindIII/AseI fragment containing the downstream human PTHrP promoter was isolated from clone SH1. The ends were blunt with dNTPs and klenow and the fragment ligated into the end-filled XbaI site in the polylinker of CATM54PA to generate -2673PTHrPCAT. To generate deletion constructs of the PTHrP promoter, the HindIII/AseI fragment was digested with XbaI, NsiI, AvrII, and AccI. Each fragment was gel isolated and ligated into CATM54PA. The deletion constructs were named -1498PTHrPCAT, -862PTHrPCAT, -329PTHrPCAT, and -45PTHrPCAT, respectively to indicate the length of the 5’ DNA relative to the exon 2 start site. All constructs were sequenced to confirm their identity by polymerase T7.

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D.P. is supported by National Institutes of Health (NIH) Grants No. DK02023 and DK47292. J.D.R. is supported by NIH Awards CA52410, CA 53632, CA 32737; NIH Shanna Award CA 04718; and USAMRDC Contract No. DAMD 17-91-C-1001.

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Blood. Vol 81, No 4 (February 15), 1993: pp 1017-1024

Blood
A p50/p65 NFκB fusion protein expression plasmid was as previously described. The IL2RαCAT29 HTLV-I LTRCAT,29 and c-jun expression vectors30 were previously described.

**Transfection and CAT assays.** Plasmid DNA used for transfection was prepared on two CsCl gradients. MLA 144 T cells, 729 B cells, and 729pH6neo cells (5 × 10⁶) were transfected in duplicate by electroporation (Biorad Laboratories, Richmond, CA) with the indicated amounts of plasmid at 250V and 960 μF in 250 μL of 1.2x RPMI 1640 medium with 10% FCS.31 Rat 1 cells were transfected in duplicate in OPTI-MEM-1 medium using the same conditions. The plasmid BC12 was added as required to standardize the total amount of DNA added to the cells. After electroporation, cells were incubated in medium containing 10% FCS for 48 hours, and then harvested into 0.01 mol/L phosphate-buffered saline. Cell membranes were disrupted by three cycles of freeze-thawing, and protein concentrations determined by the method of Bradford.32 Equal amounts of protein extract were incubated with [³⁵S] chloramphenicol as described.33 Acetylated and nonacetylated spots were cut from the chromatography paper and quantitated by liquid scintillation counting. Data are presented as percentage of acetylation.

**Northern blot analysis.** Total RNA was extracted by the guanidinium-thiocyanate method.34 Total RNA (60 μg) was separated by electrophoresis through 1% agarose gels, transferred to nitrocellulose membrane, and hybridized with [³²P]labeled cDNA probes (PTHrP, γ-actin) prepared by the method of random priming. Membranes were washed and exposed to film at -70°C with intensifying screens.35

**RESULTS**

**Transactivation of the human PTHrP promoter by HTLV-I and HTLV-I Tax** HTLV-I-infected T cells constitutively express PTHrP, suggesting that Tax might transactivate the PTHrP promoter.30 To test this possibility, human PTHrP promoter deletion mutant CAT (Fig 1A) constructs were transiently cotransfected using electroporation together with an expression vector for human Tax (BC3.9 sph) or HTLV-II Tax (BC20.2 sph). Using gibbon T cells (MLA-144) as the recipient cell line, HTLV-I Tax (BC3.9 sph) and HTLV-II Tax (BC20.2 sph) transactivated all the deletion mutants (Fig 1B). The highest levels of induction were seen with the P2673PTHrPCAT and P1498PTHrPCAT constructs. All PTHrP promoter CAT deletion mutants were similarly transactivated by HTLV-I Tax and Rex and HTLV-II Tax/Rex (data not shown). HTLV-I Tax was significantly more active in transactivating -45PTHrPCAT than HTLV-II Tax. Although the exact mechanism for this difference needs to be examined, differential transactivation of the human EGR-1 promoter by HTLV-I and HTLV-II Tax has been described.36

The carboxy terminal acidic portion of Tax is required for PTHrP transactivation. Several studies of other promoters indicate that Tax may transactivate viral or cellular gene expression through induced interactions with either NFκB or cAMP responsive sequences (CRS).33-37 The downstream human PTHrP gene promoter contains no NFκB-like sequences or classical CRS. However, a CRE-like sequence, TGACTTCA, with seven out of eight homologous base pairs is located at +32/+40. Ruben et al38 have previously reported that amino or internal region Tax protein mutations inhibit the ability of Tax to transactivate viral and cellular genes and that deletion of the 30 carboxy terminal amino acids results in a loss of HTLV-I Tax transactivation of the IL-2Rα promoter but not the HTLV-I LTR.30 Provision of exogenous p65/p50 NFκB31 in the form of a fusion protein stimulates the IL-2Rα gene, suggesting that the NFκB pathway is mediating Tax transactivation of IL-2Rα. To examine which domains of the Tax protein might be mediating transactivation of the PTHrP promoter, Tax A984, a mutant with a 30 amino acid deletion of the carboxy terminus, was compared with wild-type Tax in cotransfection assays. As positive controls for the study, we used both the IL-2Rα promoter and HTLV-I LTR linked to CAT reporter genes. Cotransfection of LTR-ICAT with Tax A984 resulted in similar levels of transactivation as seen with the intact Tax protein expressed by BC 3.9. (Fig 2) In contrast, IL-2RαCAT activation was present with wild-type Tax (BC 3.9) cotransfection, but absent with Tax A984. Cotransfection of 1L-2RαCAT with the p65/p50 NFκB expression vector resulted in marked induction of IL-2Rα promoter activity (Fig 2). When -2673PTHrPCAT activation was tested, the Tax A984 deletion mutant showed a marked decrease in transactivation compared with wild-type Tax. In contrast to the effects of NFκB on IL-2RαCAT, provision of exogenous NFκB was unable to rescue -2673PTHrPCAT expression. Tax A984 also failed to transactivate the PTHrPCAT promoter deletion mutants to similar levels seen with wild-type Tax (Fig 3).

**c-jun transactivates the human PTHrP promoter.** Constitutive expression of the c-jun transcription factor has recently been reported in HTLV-I infected cells.30 Because the PTHrP promoter contains AP-1 recognition sequences, it is possible that Tax may transactivate the PTHrP promoter via induction of c-jun. To test this possibility, cotransfection studies with HTLV-I Tax and a c-jun expression vector were performed (Fig 4). c-jun (0.5 to 4 μg) augmented the already marked Tax induction of -2673PTHrPCAT expression, whereas c-jun alone induced -2673PTHrPCAT expression by greater than 100-fold. This potent induction of -2673PTHrPCAT activity by c-jun, in addition to the additive effect seen with Tax, suggests that Tax and c-jun transactivation of PTHrPCAT may involve the same pathway. Cotransfection of -2673PTHrPCAT and c-jun into MLA T-cells, 729 B-cells, or Rat 1 cells is shown in Fig 5. The HTLV-I LTR served as the positive control. Interestingly, marked c-jun transactivation of -2673PTHrPCAT was observed in T cells with no induction in fibroblasts. c-jun cotransfection produced a small induction of -2673PTHrPCAT expression in 729 B cells. 729 B cells constitutively express c-jun (data not shown) so that additional exogenous c-jun would not be expected to produce a marked increment in PTHrPCAT expression. c-jun transactivation of the HTLV-I LTR was observed in T cells, fibroblasts, and in B cells.

**Tax transactivation of the PTHrP promoter requires a lymphoid-specific factor.** Because the PTHrP gene is expressed in many different tissues,7 we tested whether the stimulatory effect of Tax is cell specific. -2673PTHrPCAT was transiently cotransfected together with HTLV-I Tax into 729 B cells or a rat fibroblast cell line. As a positive control, the HTLV-I LTR, which is known to be transactivated by Tax in a variety of cell types, was similarly cotransfected with Tax. In both the rat fibroblast-cell line (Rat 1) and human B-cell line 729 (Fig 6), no induction of PTHrP promoter
activity was observed with HTLV-I Tax cotransfection. Cotransfection of all the PTHrPCAT deletion mutants and HTLV-I Tax into 729 B cells did not show an induction of CAT activity by Tax, showing that progressive 5' deletion of the promoter cannot overcome the apparent requirement for a cell-specific factor. Northern blot analysis indicates that none of these cell lines express PTHrP in the basal or phorbol ester–treated state (Fig 7). In contrast, MLA 144 gibbon T cells and Jurkat T cells expressed PTHrP as a consequence of Tax. In 729 pH6neo cells, which are B cells productively infected with the HTLV-II virus and in which HTLV-II Tax is constitutively produced, no PTHrP mRNA was detected; Tax also failed to transactivate the PTHrP promoter in transient cotransfection assays (data not shown). These data support the notion that Tax-mediated activation of PTHrP gene expression is restricted to T lym-
Fig 2. Transactivation of −2673PTHrPCAT, 112RocCAT, and HTLV-I LTRCAT by HTLV-I Tax/Rex, Tax A984, and NFKB. MLA T cells were electroporated with the same indicated DNA as those in Fig 1B. Results represent the mean of three experiments. Values above the bars are the fold induction.

Fig 3. Effect of the HTLV-I Tax protein and Tax A984 mutant on PTHrP deletion constructs. MLA T cells were electroporated as described. Data are from a representative experiment performed independently three times. Values above the bars are the fold induction.

Fig 4. Transactivation of −2673PTHrPCAT by HTLV-I Tax and c-jun cotransfection. MLA T cells were transfected with −2673 PTHrPCAT (8 μg), HTLV-I Tax (4 μg), and the indicated amounts of c-jun. Results represent the mean of two experiments.

DISCUSSION
Patients with humoral hypercalcemia of malignancy exhibit signs of abnormal calcium metabolism: increased renal tubular resorption of calcium, elevated nephrogenous cyclic AMP, and increased bone resorption. Although IL-1, a cytokine, was initially proposed to be a causative factor of the hypercalcemia because of its osteoclast-activating properties, the metabolic derangements that resemble hyperparathyroidism are not explained by IL-1. Furthermore, tumor necrosis factor β (TNF-β), a cytokine with bone resorbing activity has also been postulated to contribute to the hypercalcemia of ATL. However, not all tumor cells of ATL produce TNF-β and TNF-β levels may be normal in patients with acute ATL. Fresh leukemic cells isolated from patients

phocytes and may require the presence of a T-lymphoid-specific factor(s).
with ATL constituently produce transforming growth factor 
\( \beta_1 \) (TGF-\( \beta_1 \)) mRNA. Moreover, the TGF-\( \beta_1 \) promoter has been shown to be transactivated by the HTLV-I Tax protein. In vitro, exogenous TGF-\( \beta_1 \) has been shown to induce rat PTHrP in keratinocytes. Significant expression of the PTHrP gene has been detected in all patients with ATL; therefore, PTHrP is a leading candidate hypercalcemic factor in ATL. Recently, HTLV-I Tax has been shown to transactivate PTHrP via Jun/AP-1. These studies were performed to elucidate the mechanisms whereby Tax might also contribute to the pathogenesis of hypercalcemia in ATL, by direct stimulation of PTHrP in T cells.

Both HTLV-I and HTLV-II Tax proteins transactivate the downstream PTHrP promoter despite the absence of NFkB and classical CRE motifs. The longest construct, \(-2673\)PTHrP\( \beta \)-CAT, showed the greatest induction by the Tax proteins. However, this function of the wild type Tax proteins was found to be cell type-specific because it was only present in transfected T cells and not in B cells or fibroblasts. In contrast, the HTLV-I LTR was transactivated by Tax in all cell types tested. Furthermore, Northern analysis indicates that only productively HTLV-I-infected T cells express PTHrP mRNA and that even when B cells are productively infected with HTLV-II (729 pH6neo), the cells still fail to express PTHrP mRNA. These data suggest that the cell-specific effects on PTHrP may also relate to T-cell-specific transformation. This is similar to the interaction of Tax with the granulocyte-macrophage colony-stimulating factor (GM-CSF) promoter, which is also cell-type specific both in vitro and in vivo in transgenic mice and differs from Tax effects on the HTLV-I LTR.

The proto-oncogene c-jun significantly transactivated the PTHrP promoter with further augmentation by Tax. Both Tax and c-jun transactivated PTHrP efficiently in T cells. Hence, it is possible that Tax transactivation may be working via c-jun induction. Studies with HTLV-I LTR show that induction by \( \nu \)-jun can occur through the Tax responsive 21-bp repeats. However, whereas these studies suggest that Jun/AP-1 can activate the LTR, jun is not necessary for LTR transactivation by Tax.

The carboxy terminal domain of the Tax protein but not NFkB is required for PTHrP transactivation. Tax \( \Delta 984 \) abrogates PTHrP-promoter-induction and, unlike the 1L2R\( \alpha \) promoter, exogenously provided p65/p50 NFkB fails to rescue PTHrP gene expression. Therefore, Tax transactivation of PTHrP involves multiple protein-protein interactions with the acidic carboxy terminal region, but these interactions probably differ from those involved in transactivation of the 1L-2R\( \alpha \) promoter. Hence, the defect in Tax \( \Delta 984 \), although related to failure of NFkB induction in the case of the 1L-2R\( \alpha \) promoter, probably involves another factor in the case of PTHrP.

The PTHrP promoter contains a “CGCCCCCCGC” motif that has been identified as an EGR-1 binding site. The EGR-1 gene encodes a zinc finger containing transcription factor involved in growth and differentiation of numerous cell types. EGR-1 is constitutively expressed in HTLV-I
and HTLV-II transformed cell lines, and aberrant expression of EGR-1 in response to Tax might bypass the signals that normally induce T-cell proliferation and contribute to T-cell transformation. The product of the Wilm’s tumor locus is also a zinc finger containing protein that binds to the EBS (EGR binding site). Mutations of the Wilm’s tumor locus protein may result in its deficient binding to the EBS and lead to deregulated expression of genes containing this element. Numerous potential Ets-1 binding sites, characterized by the GGAA/T motif, are also present on the downstream PTHrP promoter. Ets-1 is a nuclear chromatin-associated DNA binding protein that is highly expressed in lymphoid cells. An Ets-responsive sequence (ERR-I) containing 2 DNA binding sites, ERE-A and ERE-B, on the HTLV-I LTR has been shown to mediate part of the transcriptional response to Tax. The role of the Ets-1 protein and the EBS as pertains to PTHrP gene expression needs to be elucidated. It is possible that Ets-1 contributes to the cell specificity which we observed.

Infection of permissive host cells with HTLV-I appears to induce transformation by a novel mechanism because Tax does not encode a known oncogene product. Tax induces neoplastic transformation of rat embryo fibroblasts in cooperation with H-Ras. Furthermore, mutant Tax proteins, which loose the ability to induce NFkB/rel but retain the ability to transactivate the HTLV-I LTR and c-fos promoter, mediate transformation of rat 2 fibroblasts. Because Tax modulates the expression of many cellular genes, this may contribute to its transforming ability and to the diverse clinical manifestations observed following retroviral infection. The cell-specific interaction of the PTHrP promoter with the Tax proteins will provide insight into the mechanisms of hypercalcemia in ATL, and possibly allow for the design of targeted drug therapy.

ACKNOWLEDGMENT

The authors thank V. Baichwai for the c-jun expression plasmid, D. Goltzman and G. Hendy for clone SH1, and C. Ramirez for secretarial assistance.

REFERENCES

24. Yoshimura T, Fujisawa JI, Yoshida M: Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-I: All contain a leucine zipper structure and basic amino acid domain. EMBO J 9:2537, 1990
38. Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201, 1980
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