A Cooperative Interaction of Human T-Cell Leukemia Virus Type 1 Tax with the p21 Cyclin-Dependent Kinase Inhibitor Activates the Human Immunodeficiency Virus Type 1 Enhancer

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Received 26 January 1996/Accepted 18 April 1996

Interactions between the Tax transactivator of human T-cell leukemia virus type 1 (HTLV-1) and a cell cycle regulatory protein have been examined. We report cooperative stimulation of human immunodeficiency virus type 1 gene expression by Tax and a regulator of cell cycle progression, the p21 cyclin-dependent kinase inhibitor (CKI). This cooperativity results from the effect of p21 on transcriptional coactivation by Tax-induced NF- κ B. This effect was abrogated by a mutation in Tax which specifically eliminated NF- κ B induction, was inhibitable by I κ B- α , and was markedly reduced in human immunodeficiency virus reporter plasmids with mutant κ B sites. These studies demonstrate that transcriptional activation by Tax is influenced by cell cycle regulatory proteins.

The human T-cell leukemia virus type 1 (HTLV-1) Tax protein is a transcriptional activator that not only regulates the expression of its own genome through interaction with the ubiquitous cellular transcription factor, cyclic AMP response element binding protein (CREB), but also activates the transcription of specific cellular genes (4, 9, 12, 32). Another target of Tax activation is NF- κ B (1, 18, 28), a family of Rel-related transcription factors which regulate the transcription of cellular genes important to the organism's response to stress and infection (10, 31). Recent elegant studies to address the mechanism of Tax-induced NF- κ B activation show that Tax subverts the physiologic pathways signaling through critical serine residues of I κ B- α normally required for NF- κ B induction in response to extracellular stimulation (13, 14, 16, 30, 36). Activation of NF- κ B has been utilized by a number of viruses, including human immunodeficiency virus type 1 (HIV-1), which contains two binding sites for NF- κ B in its long terminal repeat (LTR) that are required for inducible HIV gene expression. Previous studies have shown that there is variability in the ability of Tax to stimulate gene expression controlled by the HIV-1 LTR. For example, although it has been shown that Tax transactivates the HIV enhancer in transiently transfected cells (2, 33, 34), a number of groups have failed to observe this effect both in HTLV-1-infected cell lines and transient transfection studies with the full-length HIV LTR (2, 33, 35, 42). These differences are often observed despite the ability of Tax to induce NF- κ B DNA binding activity in these cells (1, 4, 15, 18, 19, 28, 38).

Another potential mechanism to activate NF- κ B-dependent gene expression is through coactivators for this transcription factor. Recent studies have raised the possibility that the p21 cyclin-dependent kinase (Cdk) inhibitor (CKI) may function as a transcriptional coactivator for NF- κ B (26), suggesting that NF- κ B transactivation may be affected by progression through the eukaryotic cell cycle. The p21 CKI is induced by the tumor

suppressor gene p53 in response to DNA damage (8) and is believed to play an important role in the control of progression through the cell cycle (11). p21 is a regulator of cyclin/Cdk activity and is capable of mediating cell cycle arrest at the G₁/S boundary (5, 7). In light of this finding of an interaction between NF- κ B and p21, the variable response of the HIV enhancer to stimulation by Tax, and previous reports of interactions between viral gene products and effectors of the eukaryotic cell cycle (20, 23, 39), we have investigated the effect of this cell cycle regulator on the regulation of gene expression by Tax.

FIG. 1. HTLV-1 Tax is a relatively weak activator of HIV-1 gene expression. Jurkat T cells were transfected with 5 μ g of either HIV-CAT or HTLV-1-CAT. Where indicated, cells were cotransfected with the indicated amount of Tax expression plasmid. A Tax deletion mutant deficient in all transactivation capacity (18) was included such that each condition contained a total of 5 μ g of Tax expression plasmid. Sixteen hours posttransfection, cells were treated as indicated with either 10 or 50 ng of PMA ml^{-1} or 200 or 500 U of TNF- α ml^{-1} . Fold activation relative to the reporter-alone (plus mutant Tax) condition is shown. Cells were harvested 24 h posttransfection, and CAT assays performed as described previously (29). The results are the means of four independent transfections $\pm$ standard error. The differences noted in this figure are statistically significant for the conditions indicated with one asterisk at $P < 0.03$ or two asterisks at $P < 0.03$ by the exact Wilcoxon rank sum test. Jurkat T cells were transfected with DEAE-dextran essentially as described previously (18). The HIV-CAT and Tax plasmids have been previously described (18, 21).

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FIG. 2. Tax and p21 function cooperatively to stimulate HIV-1 gene expression in a κ B-dependent manner. (A) Jurkat T cells were cotransfected with 5 μ g of reporter plasmid (HIV-CAT, $\Delta\kappa$ B HIV-CAT, BCAT, or 3xSp1 BCAT), together with 0.5 μ g of the indicated combinations of expression plasmid encoding Tax and p21. Control plasmids were included such that each condition contained 0.5 μ g of Rous sarcoma virus expression plasmid and 0.5 μ g of Tax expression plasmid. Fold activation relative to that of the reporter-alone condition is shown. Cells were harvested after 24 h, and CAT assays were performed. Transfections and CAT assays were performed as described in the legend to Fig. 1. The results shown are the mean of at least three independent transfections $\pm$ standard error. The effect of cotransfection of expression vectors encoding Tax and p21 on gene expression from the HIV LTR is noted to be significantly greater than additive ($P < 0.001$ by analysis of variance). For HIV-CAT and $\Delta\kappa$ B HIV-CAT, $n = 7$. For 3xSp1 BCAT, $n = 4$. The BCAT, 3xSp1 BCAT, and mutant HIV-CAT reporter plasmids have been previously described (21, 24). (B) Jurkat T cells were transfected as described for panel A. Where indicated, 2.5 μ g of expression vector for a signal-defective mutant I κ B- α in which Ser-32 and Ser-36 have been mutated to alanine (39a) was included. Control plasmids were included such that each condition contained 0.5 μ g of Tax expression vector and 3 μ g of Rous sarcoma virus expression vector. The data shown are representative of three independent transfections. The Rous sarcoma virus I κ B- α vector has been previously reported (6).

Differential stimulation of the HIV and HTLV-1 enhancers by Tax. To contrast activation of the HIV LTR by Tax with activation by other known stimulants of the HIV enhancer, Jurkat T-leukemia cells were transfected with an HIV reporter plasmid and either cotransfected with an expression plasmid encoding Tax or treated with phorbol myristate acetate (PMA) or tumor necrosis factor alpha (TNF- α) (Fig. 1, left). While HIV transactivation by Tax was observed, this effect was significantly lower than that observed for PMA- or TNF- α -treated cells. At the same time, consistent with previous reports (34), cotransfection of an HTLV-1 reporter with a Tax expression plasmid revealed a significantly larger degree of transactivation relative to its effect on the HIV-1 enhancer (Fig. 1, right).

Tax and p21 cooperatively stimulate the HIV LTR in a κ B-dependent manner. To determine whether a functional interaction could occur between p21 and Tax, expression plasmids encoding these two gene products were cotransfected with an HIV reporter plasmid into Jurkat T-leukemia cells. The level of p21 expressed in these cells is low, in keeping with the observation of mutation in the p53 tumor suppressor gene in Jurkat cells (3, 17) and with the increased activity observed upon transfection of a p21 expression vector (26). However,

FIG. 3. The Cdk inhibitory protein p16 does not stimulate HIV-1 gene expression when cotransfected with Tax. Jurkat T cells were transfected with HIV-CAT (5 μ g) together with the indicated combinations of expression plasmids encoding Tax, p21 (0.5 μ g), and p16 (0.5 μ g). Control plasmids were included such that each condition contained a total of 0.5 μ g of Rous sarcoma virus expression plasmid and 0.5 μ g of Tax expression plasmid. Transfections and CAT assays were performed as described in the legend to Fig. 1. The data shown are representative of three independent transfections.

cotransfection of plasmids encoding Tax and p21 resulted in stimulation of the HIV LTR which was significantly more than additive (Fig. 2). We found no evidence of a biochemical interaction between Tax and p21 and therefore investigated whether the functional interaction described is dependent upon the transactivation ability of Tax. To determine whether this effect was mediated by NF- κ B, a similar transfection was performed with an identical reporter in which the κ B sites of the HIV LTR have been mutated. Mutation of the κ B sites significantly reduced the transactivation by both p21 and Tax individually and markedly diminished the responsiveness to the cotransfected plasmids, suggesting that the synergistic effect was mediated, in large part, by the κ B regulatory elements. No activation of a minimal chloramphenicol acetyltransferase (CAT) reporter plasmid (BCAT) was observed when BCAT was cotransfected with expression vectors encoding Tax and p21. Similarly, cotransfection of Tax and p21 failed to induce cooperative activation of a multimerized Sp1 reporter plasmid (Fig. 2A). While we note the ability of p21 to activate transcription from the 3xSp1 reporter plasmid, cooperativity between Tax and p21 is retained when a κ B reporter plasmid which lacks Sp1 is used (data not shown). Thus, the cooperative effect of NF- κ B and Sp1 that we have previously described (25) is not required for the observed functional interaction of Tax and p21.

Cotransfection of an expression vector for I κ B- α inhibited the cooperative transactivation of the HIV LTR that is observed upon cotransfection of plasmids encoding Tax and p21. In accordance with previous reports (14), a signal-defective I κ B- α mutated at Ser-32 and Ser-36 was a considerably more potent inhibitor, capable of reducing the cooperative effect of Tax and p21 on the HIV enhancer to basal levels (Fig. 2B). Interestingly, although absolute levels were much reduced (>10 -fold), a significant cooperative effect between Tax and p21 was retained in the absence of κ B (Fig. 2A). Similar experiments were performed to determine the ability of the wild type and mutant I κ B- α to inhibit transactivation of the κ B element-deleted HIV LTR. Cotransfection with an expression vector encoding I κ B- α (data not shown) and, to a greater extent, the signal-defective I κ B- α mutant inhibited transactivation of this reporter by Tax and p21 (Fig. 2B). This result suggests that the low-level cooperative effect that is retained in the absence of κ B sites is mediated by NF- κ B, most probably

FIG. 4. Cooperative stimulation of HIV-1 gene expression by Tax and p21 is abolished by a specific point mutation of Tax which eliminates NF- κ B transcriptional activation. Cooperative stimulation of HIV-1 gene expression was observed for p21 in combination with wild-type Tax or the Tax mutant M47 but not with the Tax mutant M22. Jurkat T cells were transfected with 5 μ g of HIV-CAT together with the indicated combinations of Tax, M22 Tax, M47 Tax, and p21 expression plasmids. Control plasmids were included such that each condition contained a total of 0.5 μ g of Rous sarcoma virus expression plasmid and 0.5 μ g of Tax expression plasmid. Fold activation relative to that of the reporter alone is shown above each bar. Transfections and CAT assays were performed as described in the legend to Fig. 1. The data shown are representative of four independent transfections. The M22 and M47 Tax plasmids have been previously described (34). Loss of NF- κ B transcriptional activation function of the M22 Tax plasmid was confirmed by electrophoretic mobility shift assay. Likewise, the loss of CREB/ATF transcriptional activation function of M47 Tax was demonstrated by cotransfection with HTLV-1-CAT (data not shown).

through weaker protein-protein interactions with the basal transcription machinery.

Stimulation of the HIV enhancer by Tax demonstrated a dose-dependent response. This effect was specific for p21 and was not observed when p16 and Tax were cotransfected (Fig. 3). In contrast to p21, the p16 CKI causes cell cycle arrest by inhibiting the assembly of cyclins with Cdk4 and Cdk6 rather than by inhibiting their kinase activity (27). These results suggest that there is specificity among different CKIs with respect to their functional effects on Tax.

Cooperativity with p21 requires the NF- κ B transcriptional activation function of Tax. To characterize the functional domains of Tax required for this cooperative interaction, cotransfections were performed with the HIV-CAT reporter and previously described mutants of Tax which abrogate either its NF- κ B or CREB/ATF transcriptional activation function (34). Cotransfection of these plasmids with p21 revealed marked cooperativity between p21 and either wild-type Tax or the mutant CREB Tax transactivator (M47 Tax) (Fig. 4A). In contrast, no cooperativity was observed when HIV-CAT was cotransfected with expression vectors encoding p21 and a Tax mutant unable to activate NF- κ B (M22 Tax). This result demonstrates that cooperativity with p21 requires the NF- κ B transcriptional activation function of Tax.

Tax has been shown to play an important role in cellular transformation by HTLV-1 (22, 37, 40). It has been suggested that elevated NF- κ B binding activity may contribute to the oncogenic properties of Tax, possibly by disrupting the normal growth regulatory mechanisms of cellular genes (41); however, the mechanism of Tax-mediated transformation is poorly understood, and it remained possible that Tax interacted with factors influencing progression through the cell cycle either directly or mediated by other cellular factors. Here, we have investigated the possibility that interactions between Tax and cell cycle regulatory proteins influence activation of the HIV enhancer and other κ B-dependent genes. We find that the ability of Tax to stimulate HIV enhancer gene expression is dependent on the ability of Tax to induce NF- κ B and is influenced by the p21 CKI, which enhances activation further. Previous studies have shown variable effects of Tax transactivation on the HIV LTR; however, the *tax* gene product has consistently shown the ability to stimulate NF- κ B-mediated transcriptional activation of other reporter plasmids (1, 18, 28). Differences in the flanking *cis*-acting regulatory elements of these plasmids suggest that the interaction of NF- κ B with transcription factors that bind to flanking regions could ac-

count in part for the variability of Tax-mediated transactivation. In this study, a cellular factor involved in cell cycle progression and the regulation of terminal differentiation is shown to affect the ability of Tax to activate κ B-dependent gene expression. In recent studies, we have demonstrated that p21 influences transcriptional activation mediated by NF- κ B and shown that this stimulation results from an indirect interaction of RelA(p65) with cyclin E/Cdk2, mediated by the transcriptional coactivators, p300 and CBP (26). We have suggested that the p21-regulated activity of this Cdk2 complex affects the ability of NF- κ B to interact with the basal transcription complex (26). The present report suggests that Tax also interfaces with this pathway through its ability to induce NF- κ B binding activity, which, in turn, acts cooperatively with p21.

These findings provide further insight into the interactions of Tax with cellular cofactors which stimulate the HIV enhancer and may provide a mechanism to explain the apparently contradictory effects with the HIV enhancer in different cell types and in different studies. Taken together, these data support a model by which the assembly of transcriptional regulatory proteins occurs in cooperation with coactivators which promote their assembly with basal transcriptional factors to induce transcriptional activation. The effect of p21 on Tax-mediated transcriptional activation suggests that such interactions are influenced by cell cycle regulatory proteins and may play a role in modulating the effect of viral transactivators on the expression of other viral and cellular genes. Potentially, induction of such growth regulatory genes as interleukin-2 and interleukin-2 receptor by Tax in combination with cell cycle regulatory proteins could contribute to the transforming activities of HTLV-1.

We thank Donna Gschwend for secretarial assistance, Nancy Barrett for preparation of figures, Greg Hannon and David Beach for p21 and p16 plasmids, and Jonathan Betts and other members of the Nabel laboratory for helpful advice and comments.

N.D.P. is the recipient of a Scholar award from the American Foundation for AIDS Research (AmFAR) made in memory of James Hoese. This work was supported in part by a grant from the National Institutes of Health (RO1 AI29179 [G.J.N.]).

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