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Biochemistry

Human T-cell leukemia retrovirus-Tax protein is a repressor of nuclear receptor signaling

(retinoid X receptor transcription/cAMP enhancer binding protein/activating transcription factor/NF-κB/promyelocytic/nuclear bodies)

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ABSTRACT **The Tax oncoprotein promotes cellular transformation and is associated with the pathogenesis of adult T-cell leukemia. Tax expression activates transcription via the cAMP enhancer binding protein/activating transcription factor (CREB/ATF) and NF-κB pathways. In contrast to its positive action, here we demonstrate that Tax is a potent repressor of steroid and retinoid receptor transcription. The Tax protein becomes localized in the promyelocytic (PML) oncogenic domain, and unexpectedly, expression of the PML protein reverses Tax-induced repression. These results suggest that PML and Tax may act in opposing manners to influence nuclear receptor transcription and human T-cell leukemia retrovirus pathogenesis.**

The human T-cell leukemia retrovirus (HTLV-I), first isolated in 1980, is associated with adult T-cell leukemia and neurological diseases, such as tropical spastic paraparesis (1–6). The 40-kDa HTLV-I zinc finger protein, Tax, has been extensively studied and shown to play an important role in cellular transformation and HTLV-induced leukemia (7). The HTLV-I Tax protein is essential for viral replication and is a potent transactivator of both the HTLV and HIV-1 long terminal repeats (8–13). In addition, several cellular genes are up-regulated by Tax overexpression, including IL-2, IL-2 receptor α-chain, c-fos, granulocyte-macrophage colony-stimulating factor, and several immediate early response genes (14–22).

The HTLV-I Tax protein possesses oncogenic properties. In transgenic mice Tax expression leads to thymic aplasia, neurofibromas, and exocrinopathies involving salivary and lacrimal glands (23, 24). *In vitro*, Tax promotes transformation of human primary lymphocytes and rat primary fibroblasts, possibly via activation of NF-κB (25–27). It has been postulated that Tax activates NF-κB through induced degradation and/or dissociation of cytoplasmic complexes containing p105 or p100 and/or induced phosphorylation-degradation of IκB proteins, resulting in p65 translocation to the nucleus (28–32). Because Tax lacks known DNA binding activity (33–35), it is unclear how it coordinates its multiple transcriptional functions, particularly with regard to its ability to promote oncogenesis. Recently, Tax was shown to associate with the cAMP enhancer binding protein (CREB) binding protein (CBP), a coactivator molecule required for CREB/activating transcription factor (ATF)-dependent transcription of the HTLV promoter (36). In this case, Tax appears to function as an adaptor between CREB and CBP, promoting the formation of a tripartite CREB-CBP-Tax complex that bypasses the need for cAMP for activation (36).

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The nuclear receptor (NR) superfamily includes the steroids, thyroids, retinoids, and peroxisome proliferator-activated receptors (PPARs) (37, 38). Steroids and retinoids are widely reported to exert antiproliferative and anti-inflammatory effects in T lymphocytes, in part by repressing transcriptional activities of immediate early genes, such as *AP1* and NF-κB (39–43). For example, nuclear receptors such as the glucocorticoid receptor (GR) and retinoid X receptor (RXR) have been shown to "cross-couple" with *AP-1* or NF-κB signaling pathways, potentially by sequestering coactivators required for *AP1*/NF-κB transcriptional activities. Cross-coupling is likely to be physiologically important as inhibition of NF-κB by activated GR has been suggested to play a key role in glucocorticoid-mediated anti-inflammatory responses (43–45).

We recently reported that the activity of certain NRs is dramatically potentiated in the presence of overexpressed promyelocytic (PML), a protein associated with acute promyelocytic leukemia (APL) (46, 47). The PML gene encodes for a widely expressed protein that carries a ring finger and a coiled-coil motif (48, 49). PML and several associated proteins are localized to a particular subnuclear compartment known as ND10, Kr bodies, and the PML oncogenic domains or PODs (refs. 48, 50, and 51; for review see ref. 46). APL patients harbor a t(15;17) translocation that fuses the PML nuclear protein to retinoic acid (RA) receptor α and is the apparent etiologic agent of the transformation (46). We recently demonstrated that the cofactor CBP physically associates with PML and that both proteins are colocalized in the PML nuclear domain *in vivo*, suggesting a direct association between CBP and PML functions (47, 51).

As part of our studies of NRs and their regulatory role in inflammatory diseases, we asked whether Tax is able to modulate NR transcription. Indeed, we show that Tax is a potent inhibitor of GR, RXR, and PPAR target genes and, surprisingly, that PML expression is able to restore NR signaling by blocking the Tax effect. These data suggest that the proinflammatory effects of the Tax oncoprotein in part involve inhibition of NR coactivator functions and provide further evidence for a role of PML and its associated nuclear body in hormone action.

MATERIALS AND METHODS

Plasmids. Apol4-tk-Luc, CRE5-tk-Luc, and Igκ3-tk-Luc reporter genes contain the indicated number of synthetic

Abbreviations: HTLV, human T-cell leukemia retrovirus; CREB, cAMP enhancer binding protein; CBP, CREB binding protein; NR, nuclear receptor; GR, glucocorticoid receptor; RA, retinoic acid; RXR, retinoid X receptor; PML, promyelocytic; POD, PML oncogenic domain; PPAR, peroxisome proliferator-activated receptor; CMV, cytomegalovirus; TPA, phorbol 12-tetradecanoate 13-acetate; ATF, activating transcription factor.

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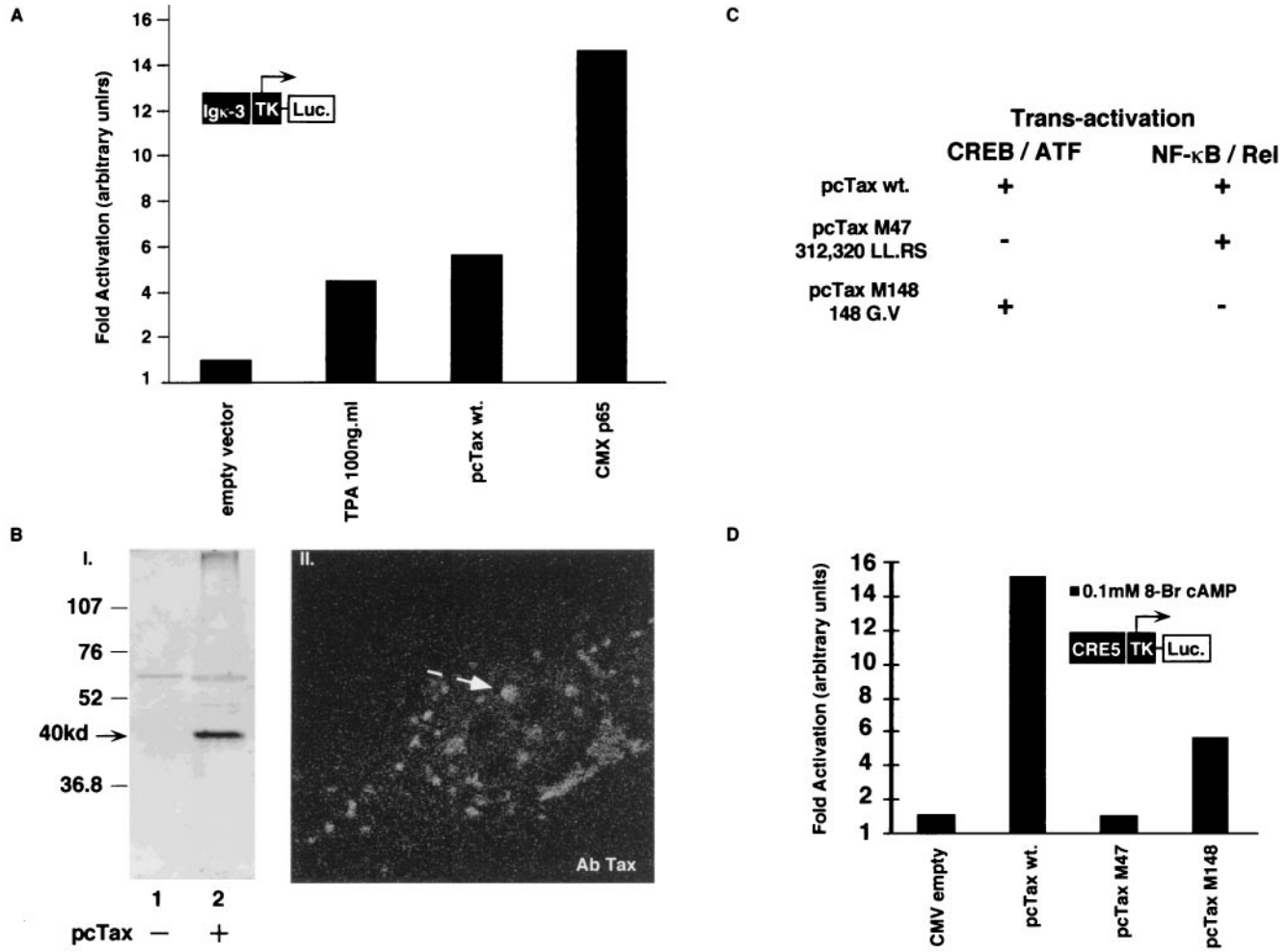

FIG. 1. Tax expression and positive transcriptional regulation. (*A*) Tax activates NF-κB. CV1 cells (48-well plates) were transiently transfected with 120 ng of reporter construct Igκ3-tk-Luc, 90 ng of CMXβgal, 120 ng of CMX-p65, and 180 ng of pcTaxwt., as indicated. All the transfection points were equalized with CMV empty vector. Transfected cells were treated with 100 ng/ml of TPA for 8 hr before the assay, where indicated. All points were performed in triplicate and varied by less than 10%. The presented values correspond to an average experiment of at least four independent assays. (*BI*) Immunoblotting of total CV1 cell extracts transfected with empty (lane 1) and pcTax (lane 2) expression vectors at 4 μg, respectively. Cell extracts were analyzed in a 12% SDS gel, transferred to a nitrocellulose membrane, and probed with the Tax polyclonal antibody. The arrow shows the 40-kDa Tax protein. (*BII*) Immunofluorescence analysis of CV1 cells (confocal microscopy), transfected at 70% confluence with CMXTax expression vector at 4 μg. Cells were grown in the absence of any ligand and fixed 12-hr posttransfection. Tax expression in the cell was revealed with the primary Tax mAb and visualized with a secondary antibody conjugated to fluorescein isothiocyanate. The arrowhead shows Tax expression in the nucleus localized in speckled-like domains. (*C*) Schematic representation of Tax wild type and mutants and their effects in CREB/ATF and NF-κB pathways. + and − indicate relative activity of expression vectors, respectively. (*D*) Tax activates a CRE response element. Transient transfection was conducted as in *A*. The CRE5-tk-Luc and Tax expression vectors are supplied at 120 ng and 180 ng, respectively. Cells were treated with 8-Br-cAMP (8-bromo-adenosine-3′:5′-monophosphate, cyclic monosodium salt) at 100 μM for 4 hr before harvesting.

oligonucleotides linked to the minimal thymidine kinase promoter upstream of the coding sequence for the luciferase gene. The oligonucleotides for Apol4-tk-Luc, CRE5-tk-Luc, and Igκ3-tk-Luc, respectively, correspond to the A site of the apolipoprotein AI promoter (47), the CRE element of the somatostatin promoter (52), and the NF-κB site of the Ig κ light enhancer core element (27). The (−520)aP2-Luc. reporter contains the promoter region of the *aP2* gene (−520 nt) (53) upstream of the luciferase gene (kindly provided by P. Tontonoz, Salk Institute La Jolla, CA). MMTV-Luc, Gal4-tk-Luc, CMXβgal, CMX-RXRα, and CMXPML have been described (47). CMX PPARγ has been described (54). CMX Gal-4RXRαF contains the full-length mouse RXRα coding sequence in-frame with the Gal-4 DNA binding domain downstream of the cytomegalovirus (CMV) promoter. CMX-Tax contains the full-length viral Tax HTLV-1 coding sequence downstream of the CMV promoter. pcTax wt. and pcTaxM47 (33) and pcTaxM148 (27) are driven by the CMV promoter.

Cells. CV1 and Hela cells were maintained as monolayers in DMEM supplemented with 100 units/ml of penicillin-streptomycin and 10% resin-charcoal-stripped (55) bovine calf serum or 10% FCS, respectively, (GIBCO). Cultures were maintained at 37°C and in 7% CO_2. For immunofluorescence, cells were grown on round coverslips (Corning) in 6-well plates.

Antibodies and Reagents. mAb Tax 1315 (American Tissue Culture Collection) or an anti-Tax polyclonal serum were used in the immunofluorescence and Western blot analysis to detect the Tax protein. 8-Br-cAMP (8-bromo-adenosine-3′:5′-monophosphate, cyclic monosodium salt) was used at 100–200 μM. Phorbol 12-tetradecanoate 13-acetate (TPA; 100 ng/ml), 10 nM-1 μM dexamethasone, 10 nM-1 μM 9-cis RA (Sigma), 0.1–100 nM LG69 (Ligand Pharmaceuticals, La Jolla, CA), and 5 μM BRL49653 (Biomol, Plymouth Meeting, PA) were dissolved in organic solvents and applied in less than 0.1% of the media volume.

Transfection. For transient transfections, CV-1 and Hela cells were grown in 6- or 48-well plates to 50–80% confluence

in the corresponding medium. Twelve hours later the cells were transiently transfected with the indicated expression vectors by lipofection using *N*-[1-(2,3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethyl ammonium methyl sulfate] (DOTAP) according to the manufacturer's instructions (Boheringer Mannheim). Cells were analyzed for the expression levels of different proteins and the localization of the overexpressed and/or endogenous proteins in the nuclei.

Reporter Assays. Luciferase and β-galactosidase were assayed as described (47). Extracts were prepared 24–30 h after transfection except otherwise indicated. Equal quantities of extract protein were assayed in each point. Results are given as a relative activity, based on a positive control arbitrarily set at 1, observed in each described experiment in the absence of coactivators and/or ligands.

Immunohistochemistry. Cells were fixed as described (55). Fluorescence images were analyzed in confocal microscopy. For the *in vitro* immunodetection assay extracts were analyzed in a 12% SDS gel, transferred to a nitrocellulose membrane, and probed with the corresponding antibodies as described (56).

RESULTS

Tax Expression Activates the NF-κB and the CREB/ATF Pathways. In agreement with previous studies (27, 33), transient expression of Tax induces transcriptional activation of an NF-κB-regulated target gene (Fig. 1*A*) that is equipotent to TPA treatment and 50% as efficient as transfection of a p65 expression vector. Tax stimulation of NF-κB transcription occurs by enhancement of the binding of p65 protein to NF-κB response elements within the promoter region of reporter genes (16, 29, 31, 32). In CV1 cells, transient transfection of a Tax expression vector produces the expected 40-kDa protein (Fig. 1*BI*), which is localized in both the nucleus and the cytoplasm in diffuse and speckled-like domains (Fig. 1*BII*). In the nucleus, Tax is embedded within as well as surrounding these nuclear speckled-like structures (Fig. 1*BII,* see arrowhead and data not shown). Previous studies have shown that Tax binds the Int-6 protein, which is a known component of the PML nuclear body (57).

We next analyzed Tax mutations that selectively activate either the NF-κB or the CREB/ATF transcriptional pathways. As illustrated in Fig. 1*C*, the TaxM47 mutation (Leu-319 and Leu-320 to Arg and Ser) (32) and TaxM148 (Gly-148 to Val) (27) activate only the NF-κB/Rel or CREB/ATF pathways, respectively. In agreement with previous studies (36), transient expression of wild-type Tax activates a cellular CRE containing reporter gene in the presence of elevated cAMP (Fig. 1*D*). As expected, M148 also activates CRE5-tk-Luc whereas M47 was inactive for CREB/ATF-dependent transcriptional activation (Fig. 1*D*). Similarly, Tax wild type and M47 selectively activate transcription from an NF-κB responsive promoter (Fig. 1*A* and data not shown).

Tax Represses GR-Dependent Transcription. Because Tax induces inflammatory responses in T lymphocytes, we reasoned that it also may influence the action of NR's implicated in anti-inflammatory activities. Indeed, Tax expression in Hela cells severely represses hormone-induced GR transcription, in a concentration-dependent manner (Fig. 2*A* and data not shown). Surprisingly, the M148 mutation that selectively activates CREB/ATF, but not the NF-κB pathway, was equipotent to Tax in repressing GR. In contrast, whereas M47 retains some repressive activity, it is significantly less effective than wild-type Tax. Taken together the above demonstrate that effective repression is linked to CREB/ATF activation.

Because NF-κB activation is known to repress GR target genes (43), the results with mutant M148 on GR suggested that the biochemical mechanism of Tax induced NF-κB transactivation may be different from the one responsible for NF-κB/GR cross-repression. Therefore, we examined the potential cooperative effect of Tax and RelA/p65 on GR function. In a transient transfection assay Tax and p65 cooperate to virtually completely repress GR transcription (Fig. 2*B*). Similarly, treatment of the cells with TPA also cooperates with Tax (Fig. 2*B*, open bars) to increase repression. These results suggest a likely independence of Tax and NF-κB repression pathways. Mutations in the ligand binding domain, which

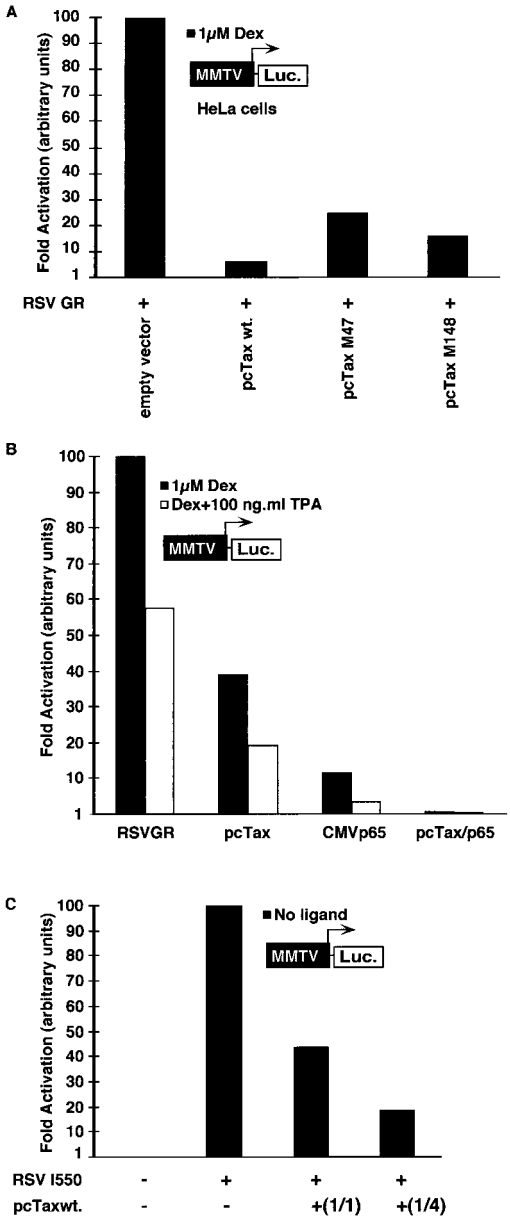

FIG. 2. Tax represses GR receptor activity. (*A*) Tax wild-type and mutant vectors repress GR in Hela cells in the presence of 1 μM of dexamethasone (Dex). Transient transfection was conducted as described in Fig. 1*A*. MMTV-Luc was transfected at 120 ng, pcTax at 180 ng, and the glucocorticoid expression vector RSVGR at 90 ng, respectively. All of the transfection points were equalized with CMV empty vector. Transfected cells were treated with 1 μM Dex for 12 hr before the assay. (*B*) RelA/p65 or TPA cooperate with Tax to repress GR. Experiment is done as in *A* but using CV1 cells. CMX-p65 is used at 90 ng, and cells were treated with ligands for 12 hr before the assay, as indicated. (*C*) Tax represses I550 transcription. CV1 cells were transfected as in *B*. RSVI550 is used at 120 ng, and the pcTax was transfected at the indicated molar ratio with I550. All transfections points were equalized for the total amount of transfected plasmids with a CMV empty vector.

constitutively activate the GR (I550), are still repressed by Tax although it is notably less effective (Fig. 1*C*), indicating that it acts on both amino terminal (Tau-1/AF-1) and carboxyl terminal (AF-2) activation functions.

Tax Represses RXR and PPAR Activities. We next examined the potential regulatory effect of Tax on other members of the NR superfamily. As shown in Fig. 3*A*, Tax, M148, and M47 suppress 9-*cis* RA activation of RXR, although the M47 effect strongly depended on growth conditions (data not shown). Activation by the RXR-specific ligand LG69 also was blocked (data not shown).

PPARγ and RXRα form a heterodimeric complex that functions as a central regulator of signaling pathways involved in such as the adipocyte diffrentiation (53). It has been shown that specific ligands for PPARγ and RXRα activate in a receptor-dependent manner the expression of terminal adipocyte differentiation-specific marker genes, such as the *aP2* (53, 54). In CV1 cells transient transfection of PPARγ and RXRα activated the (−520)aP2-Luc reporter gene 5- to 7-fold after addition of ligand (Fig. 3*B*). As expected, Tax expression repressed significantly both 9-*cis* RA and the PPARγ-specific ligand BRL49653 activation of the *aP2* promoter, further supporting the notion that Tax functions as a general NR repressor. These results suggest that Tax might be a general inhibitor of ligand-induced NR activation and thus could block anti-inflammatory effects of glucocorticoids, retinoids, and possibly other nuclear hormones.

PML Cellular Protein Opposes Tax. It has been shown that PML expression stimulates the transcriptional activity of NRs such as the progesterone, glucocorticoid, and RXRs (46, 47, 58). The biochemical basis for this phenomenon has been attributed to the physical association of PML, CBP, and p300 and is supported by the observed compartmentalization of CBP to PML nuclear bodies (PODs) (47, 51). Because PML potentiates NR transcription, and because Tax can be found in PODs, we asked whether PML can reverse Tax-induced repression of GR activity. Indeed, PML coexpression (1:1 molar ratio) results in a substantial reversal of Tax- and M47-induced repression of the mouse mammary tumor virus promoter (Fig. 4*A*). In addition, expression of PML at increasing concentrations completely restores GR transcription to wild-type or higher levels of activity (Fig. 4*B*).

The effect of Tax on NRs was further analyzed by using a modified "one-hybrid" assay based on transfection of a Gal-4 DNA binding domain/RXRα fusion (Gal-4 RXRα F). Al-

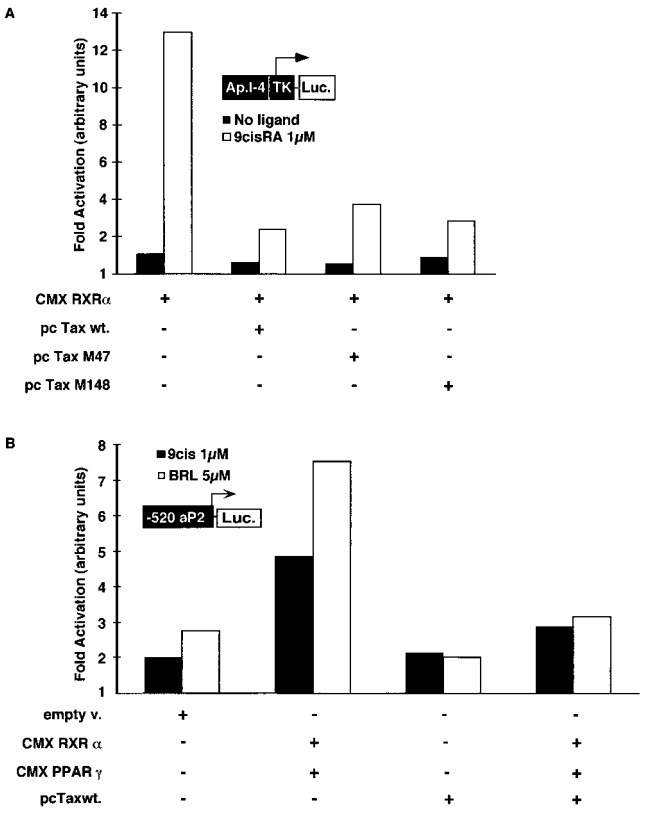

FIG. 3. Tax represses RXRα and PPAR gamma transcription. (*A*) Tax wild type and mutants repress RXRα in CV1 cells in the presence of 1 μM 9-*cis* RA. CV1 cells were transfected as in Fig. 1*A*. Apol4-tk-Luc., CMX RXRα, and Tax expression vectors were transfected at 120 ng, 90 ng, and 180 ng, respectively. Cells were grown in the absence or presence of 1 μM 9-*cis* RA for 12 hr before the assay. (*B*) Tax represses *aP2* gene expression. CV1 cells were transfected as in Fig. 1*A*. The (−520)aP2-Luc. reporter was transfected at 120 ng. CMX RXRα and CMX PPARγ were transfected at 60 ng, respectively, and the pcTax at 120 ng. Cells were treated with 9-*cis* RA at 1 μM or the PPARα ligand BRL49653 at 5 μM for 12 hr before the assay. All points were performed in triplicate and vary by less than 10%. The presented values correspond to a representative experiment of three independent assays.

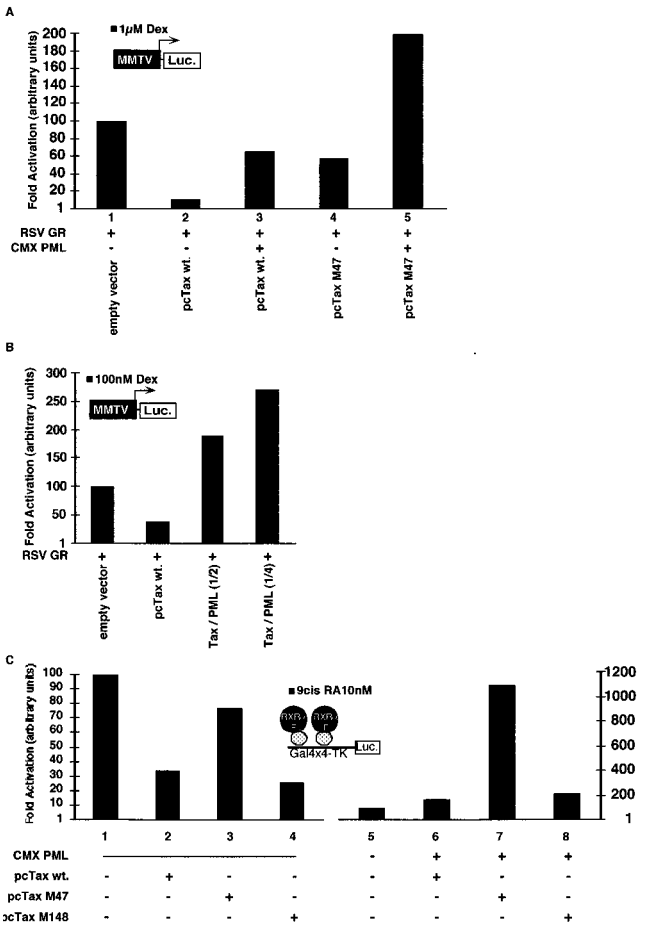

FIG. 4. PML reverses Tax repression. (*A*) PML blocks GR repression. CV1 cells were transfected as in Fig. 2*A*. CMX PML was used at 180 ng as the Tax expression vector. (*B*) Experiment was done as in *A*, but pcTax was used at 90 ng and CMX PML was transfected at the indicated molar ratio. After transfection cells are grown for 12 hr in the presence of 100 nM of dexamethasone before harvesting. (*C*) Tax represses Gal-4 RXRαF and PML restores activity. Cells were transfected as before. Gal-4 RXRαF was transfected at 120 ng and CMX PML and Tax expression vectors at 90 and 180 ng, respectively. Transfected cells were treated with 9-*cis* RA at 1 μM for 8 hr before the assay. In the histogram two bars of activity are used (100 and 1,200 arbitrary units), as the activity of the reporter is dramatically stimulated in the presence of PML protein.

though this construct possesses a relatively high basal activity, the final activation by ligand (2- to 3-fold) again was repressed by cotransfection of wild-type Tax and M148 (Fig. 4*C*, lanes 1–4 and data not shown), whereas M47 possesses poor repressor activation. Interestingly, coexpression of PML with Tax proteins at a 1:2 molar ratio not only restored but also potentiated Gal-4 RXRα activity (Fig. 4*C,* lanes 5–8). This effect of PML on Tax transrepression is most impressive in the case of M47, where PML potentiated Gal-4 RXRα 12-fold (Fig. 4*C,* compare lanes 5 and 7).

DISCUSSION

Previous studies have suggested that the Tax oncoprotein acts to stimulate NF-κB and CREB/ATF target genes, such as the HTLV long terminal repeat. Because of its role in transcriptional transactivation, Tax is proposed to serve as a positive transcriptional coactivator. In contrast, our data show that Tax modulates NR signaling by acting as a novel transcriptional repressor. This dual activity suggests Tax may more appropriately be considered a transcriptional modulator, stimulating some genes while inhibiting others. Because negative and positive regulatory processes control the interplay between cell growth vs. cell differentiation, Tax may play a more complex role than previously suggested in altering T-cell activation.

Data with Tax mutation M148, which is defective for NF-κB but not for CREB/ATF activation, show that Tax-induced NF-κB transcription might be dispensable for Tax effects on NRs. This conclusion is further supported by the coexpression of Tax and p65, which show an additive effect in repressing GR and RXR, indicating they may represent two independent inhibitory pathways. Reciprocally, results with the M148 mutant suggest that Tax activation of CREB/ATF pathway promotes NR repression.

We conclude that the Tax proteins that can activate CREB/ATF targets are effective repressors although we cannot conclude that ATF activation is required for repression. Indeed, how Tax represses remains unclear. Perhaps the strongest lead is based on the observation that PML reverses or blocks the Tax effect. Previous studies have shown that Tax can be localized to PML bodies by virtue of its association with the Int-6 protein (57). In addition, CBP binds both Tax and PML (through independent sites) (47) and CBP can clearly be localized in PODs (47). The ability of PML to block Tax repression and thus restore NR transcription suggests that PML, and by extension PODs, may be a link to the positive and negative actions of the Tax protein. This work does not establish that PODs are essential intermediates in Tax action, but along with the previous studies raises this as a distinct possibility.

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