VIROLOGY 225, 52–64 (1996)
ARTICLE NO. 0574

Human T Cell Leukemia Virus Type 1 Tax Protein Increases NF-κB Dimer Formation

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Received July 9, 1996; accepted August 27, 1996

Human T cell leukemia virus type 1 (HTLV-1) encodes a strong transcriptional transactivator, the Tax protein, that stimulates viral transcription through the long terminal repeat and also stimulates many cellular genes via the activation of host transcription factors. Previous studies have demonstrated that Tax activates NF-κB through binding to the Rel homology domain of NF-κB proteins. Tax was also shown to increase degradation of IκBα resulting in the induction of NF-κB DNA binding activity. We addressed the specificity and function of Tax interaction with members of the NF-κB/IκBα family by using EMSA, protein affinity chromatography, protein–protein crosslinking and co-immunoprecipitation assays. The results of the present study demonstrate that: (1) Tax enhances NF-κB binding to DNA 40- to 100-fold by increasing NF-κB dimer formation which can be detected in the absence of DNA; (2) Tax binds to all NF-κB DNA binding subunits *in vitro* and to IκBα; (3) Tax physically associates with IκBα *in vivo;* and (4) Tax and IκBα have antagonistic effects on NF-κB binding and gene activity. These results suggest that Tax interaction with IκBα interferes with the formation of NF-κB–IκBα complexes and may play a role in targeting IκBα for degradation.

INTRODUCTION

Human T cell leukemia virus type I (HTLV-1) is the etiologic agent of adult T cell leukemia (ATL) and several neurodegenerative disorders including tropical spastic paraparesis (Poiez *et al.,* 1980; Yoshida *et al.,* 1982, 1984). A hallmark of HTLV-1 pathogenesis is its latency; ATL develops after a characteristically long latent period of 20 or more years after initial exposure (Yoshida *et al.,* 1984), while HTLV-1 associated myelopathies manifest symptoms 3 to 5 years after infection (Sodroski, 1992). In addition to the structural proteins, HTLV-1 encodes a number of regulatory proteins, including Tax and Rex, which are involved in controlling viral gene expression and pathogenesis (Inoue *et al.,* 1987; Sodroski, 1992). The 40-kDa transactivator protein Tax is critical for modulating HTLV-1 gene expression and cellular transformation (Felber *et al.,* 1985; Sodroski *et al.,* 1985; Nyborg *et al.,* 1988; Sodroski, 1992; Hiscott *et al.,* 1995). Tax possesses transforming potential both in cultured cells (Tanaka *et al.,* 1990; Smith *et al.,* 1991; Yamaoka *et al.,* 1996) and in transgenic mice (Nerenberg *et al.,* 1987; Yamaoka *et al.,* 1996; Grossman *et al.,* 1995). The effect of Tax on gene transcription is mediated indirectly through interaction with cellular transcription factors such as CREB/ATF, SRF, and Ets, which bind to the 21-bp repeat cyclic AMP responsive elements (CRE) in the long terminal repeat (LTR) of HTLV-1 (Nyborg *et al.,* 1988; Giam *et al.,* 1989; Xu *et al.,* 1994). Tax also induces numerous cellular genes, many of which are involved in T cell activation and growth such as IL-2, IL-2Rα, and c-fos (Yoshida, 1993). Induction of at least some of these genes involves the interaction of Tax with members the NF-κB/Rel family of transcription factors.

The NF-κB/Rel transcription factors are a family of dimer-forming proteins that bind to the consensus DNA sequence 5′-GGGANNYYCC-3′. NF-κB/Rel proteins are pleiotropic regulators of cellular and viral genes implicated in immunoregulatory processes and cell growth control (Baeuerle *et al.,* 1994; Verma *et al.,* 1995; Roulston *et al.,* 1995). Structurally, all DNA-binding members of the family — NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB — share an amino-terminal rel homology domain involved in DNA binding, protein dimerization, and nuclear translocation (Baeuerle *et al.,* 1994; Verma *et al.,* 1995; Roulston *et al.,* 1995). These proteins can form homo- or heterodimers in various combinations and differentially regulate transcription of NF-κB-dependent genes (Baeuerle, 1991). In resting T cells, NF-κB/Rel proteins are sequestered in the cytoplasm by ankyrin repeat-containing inhibitory molecules that include IκBα, IκBβ, IκBγ, p105, p100, and bcl-3 (Beg *et al.,* 1993a; Verma *et al.,* 1995; Roulston *et al.,* 1995). Both IκBα and IκBβ play an important role in binding to NF-κB/Rel proteins; the nuclear localization sequence signal of NF-κB subunits is masked by IκBα, thereby preventing nuclear transloca-

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0042-6822/96 $18.00

tion (Beg *et al.,* 1992, 1993a; Verma *et al.,* 1995). Cellular stimulation by an array of activating compounds leads to the rapid degradation of both IκBα and IκBβ which in turn allows for nuclear translocation of the NF-κB/Rel dimer complex (Verma *et al.,* 1995).

The HTLV-1 Tax protein is known to strongly activate nuclear expression of NF-κB/Rel proteins (Yoshida, 1993), although the precise mechanism by which Tax produces these effects remains unclear. No NF-κB binding sites are found within the HTLV-1 LTR; therefore, NF-κB is not required for HTLV-1 gene expression. However, constitutive NF-κB activity is observed in HTLV-1 infected cells (Arima *et al.,* 1991; Kanno *et al.,* 1994a; Sun *et al.,* 1994; Yamaoka *et al.,* 1996). Stable Tax expressing rat fibroblasts displayed constitutive NF-κB binding activity which was essential for Tax mediated transformation (Nerenberg *et al.,* 1987; Kitajima *et al.,* 1992; Yamaoka *et al.,* 1996). Physical interaction occurs between Tax and the Rel homology domain of NF-κB proteins; *in vitro* and *in vivo* Tax associates with NF-κB2 (p100/p52), as well as with NF-κB1, RelA(p65), and c-Rel (Hirai *et al.,* 1992; Beraud *et al.,* 1994; Lanoix *et al.,* 1994; Pepin *et al.,* 1994; Watanabe *et al.,* 1994; Suzuki *et al.,* 1994; Murakami *et al.,* 1995; Kanno *et al.,* 1994a, b). In addition, physical interaction between NF-κB and Tax results in the release of NF-κB complexes such as c-Rel, p50, and p65 from inhibition by p100 and p105 (Watanabe *et al.,* 1993; Kanno *et al.,* 1994b; Watanabe *et al.,* 1994; Beraud *et al.,* 1994). HTLV-1 infected and Tax-expressing cells also have a higher rate of degradation of IκBα and IκBβ (Béraud *et al.,* 1994; Lacoste *et al.,* 1995; Maggirwar *et al.,* 1995; Kanno *et al.,* 1995; Good *et al.,* 1996), thereby permitting the continuous release of NF-κB/Rel and constitutive NF-κB specific DNA binding activity. Although physical association of Tax with IκBα has also been observed, the significance of this interaction remains unclear (Suzuki *et al.,* 1995). Mutations in IκBα at serine 32 and/or serine 36 are not subject to Tax-mediated degradation (Brockman *et al.,* 1995; Kanno *et al.,* 1995; Brown *et al.,* 1995), suggesting that Tax may be acting upstream of a signaling pathway or kinase(s) required for phosphorylation of IκBα and subsequent degradation (Brockman *et al.,* 1995; Kanno *et al.,* 1995; Maggirwar *et al.,* 1995).

Recent studies have demonstrated that Tax physically associates with basic leucine zipper (bZIP)-containing transcription proteins such as the CREB/ATF family (Zhao *et al.,* 1991; Wagner *et al.,* 1993) and enhances dimerization of CREB/ATF in the absence of DNA (Zhao *et al.,* 1991; Wagner *et al.,* 1993; Giovanni *et al.,* 1995; Yin *et al.,* 1996; Kwok *et al.,* 1996; Bantignies *et al.,* 1996). Tax interacts with the basic region of bZIP proteins which mediates DNA contact (Giovanni *et al.,* 1995). The enhancement of CREB dimerization results in enhanced CREB binding to specific CRE sites within the HTLV-1 LTR but not to CRE sites in the somatostatin gene promoter (Yin *et al.,* 1996; Kwok *et al.,* 1996; Bantignies *et al.,* 1996). The ability of Tax to mediate an increase in DNA binding is highly dependent on the core CRE binding sequence and its flanking elements. The CREB dimer alone is not capable of efficiently binding to the cellular CRE sequence but also requires the CREB coactivator, CBP (Kwok *et al.,* 1996).

In this study, we addressed the specificity and function of Tax interaction with members of the NF-κB/IκBα family. Using a variety of techniques, we demonstrate that: (1) Tax binds to all NF-κB DNA-binding subunits *in vitro* and to IκBα; (2) Tax enhances NF-κB binding to DNA by increasing the formation of NF-κB dimers which can be detected in the absence of DNA; (3) Tax interacts with IκBα *in vivo;* and (4) Tax and IκBα have antagonistic effects on NF-κB binding and gene activity.

MATERIALS AND METHODS

Protein expression and purification

Plasmids for the expression of NF-κB–GST fusion proteins were produced by subcloning different NF-κB cDNAs into pGEX2T or pGEX3X vectors (Pharmacia) (Lin *et al.,* 1995); p50, p65, and N′p65 (transactivation domain is deleted) were cloned into pGEX3X, and p52 and IκBα were cloned into pGEX2T. The GST fusion proteins were isolated from *Escherichia coli* DH5α strain (GIBCO/BRL) following a 3-hr induction with 1 m*M* IPTG (Pharmacia) at 37°. Bacterial extracts in PBS containing 1% Triton X-100 were incubated with glutathione Sepharose beads (Pharmacia) for 20 min at room temperature. After washing three times with PBS, the fusion proteins were eluted from the beads with 25 m*M* glutathione in 50 m*M* Tris–HCl (pH 7.5) and stored with the addition of protease inhibitors. c-Rel and Tax were polyhistidine tagged by subcloning into the pAcH6N1 vector. For the production of polyhistidine tagged protein, recombinant baculoviruses were prepared using the BaculoGold transfection kit as recommended by the manufacturer (PharMingen). Sf9 cells were infected with recombinant baculoviruses and cultured for 4 days at 28°. Infected cells were harvested, washed with PBS, and lysed in binding buffer (5 m*M* imidazole, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 7.9). Recombinant proteins were purified using rapid affinity purification with His-Bind metal chelation resin under nondenaturing conditions as recommended by the manufacturer (Novagen, pET System Manual).

Immunoprecipitations

N-Tera 2 cells (2×10^6 cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 m*M* glutamine, and 50 μg/ml gentamycin. N-Tera 2 cells were transfected by the calcium phosphate precipitate method with wild-type Tax (10 μg) or with the Tax mutants M22 (NF-κB$^-$/CREB$^+$) or M47

(NF-κB$^+$/CREB$^-$) (Smith *et al.,* 1990) and with 0–10 μg of IκBΔ4 (Lin *et al.,* 1996; Beauparlant *et al.,* 1996c) for 48 hr. C8166 cells were either treated or not treated with calpain inhibitor I (100 μM) (ICN) or with 100 μM pyrrolidine dithiocarbamate (PDTC) (Sigma) for 1 hr. Cells were lysed in TN-NP-40 buffer (20 m*M* Tris–HCl (pH 7.5), 200 m*M* NaCl, and 0.5% NP-40). Extracts were then subjected to immunoprecipitation as follows: protein (500 μg) was diluted in TN-NP-40 (total volume 50 μl). The extract was first precleared by incubation with normal rabbit serum (NRS) at a final dilution of 1:25 for 1 hr at 4°, followed by recovery of nonspecific immune reactions on protein A–Sepharose (4°, 1 hr). Polyclonal anti-Tax or anti-IκBα antiserum was then added to a final dilution of 1:25 and incubated for an additional 1 hr at 4°. The specific immune complexes were recovered with protein A–Sepharose by a final incubation of 1 hr at 4°. The beads and immune complexes were washed three times in TN-NP-40, resuspended in sample buffer, boiled, and resolved on SDS–PAGE. Gels were transferred to nitrocellulose and incubated overnight with monoclonal IκBα or Tax antibody. Peroxidase-conjugated anti-mouse antibody was used against the monoclonal antibody and the bands were visualized by the enhanced chemiluminescence detection system (Amersham).

Electrophoretic mobility shift assay (EMSA)

The HIV-1 NF-κB probe which contains two NF-κB binding sites (5′-AGGGACTTTCCGCTGGGGACTTTCC-3′) and the MHCI probe (5′-CAGTGGGGAATCCCCATAG-3′) were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The binding reactions were carried out with recombinant protein and 0.2 ng of probe in a final concentration of 10 m*M* HEPES (pH 7.9), 50 m*M* KCl. 0.1 m*M* EDTA, 0.25 m*M* DTT, 1 μg of poly(dI–dC), and 10% glycerol and the final volume was brought to 20 μl with H_2O. Recombinant proteins were incubated with the probe for up to 1 hr at room temperature; the mixture was loaded on a 5% polyacrylamide gel (60:1 acrylamide:*N,N*-methylene bisacrylamide) prepared in 1× Tris–glycine (pH 8.3) and electrophoresed at 200V for 2–3 hr. Gels were then dried and exposed on film at −80° overnight. In competition reactions a 100-fold excess of unlabeled oligonucleotide was added to the reaction mixture for 15 min prior to the addition of radiolabeled probe.

GST affinity chromatography

The GST fusion proteins were expressed and immobilized on glutathione beads as described above. The GST–NF-κB proteins immobilized onto the glutathione agarose beads were washed three times with HNTG (20 m*M* HEPES, pH 7.9, 150 m*M* NaCl, 10% glycerol, and 0.1% Triton). For protein interactions, 300 ng of glutathione agarose bound GST-NF-κB was incubated overnight at 4° with 50 ng of His-Tax in a final HNTG volume of 200 μl. Following incubation, the beads were washed three times in 500 μl of HNTG and resuspended in 20 μl of SDS sample buffer. The samples were then boiled and loaded on an SDS–PAGE for immunoblot analysis with anti-Tax antibody.

NF-κB protein crosslinking

Chemical crosslinking of recombinant NF-κB proteins was performed using 50–200 ng of NF-κB protein alone or in the presence of 50–200 ng of His-Tax. NF-κB proteins were allowed to incubate with or without Tax in binding buffer (20 m*M* HEPES, pH 7.9, 200 m*M* NaCl; 20 m*M* Tris–HCl, pH 7.5, and 1 m*M* DTT) for 30 min at room temperature. Dimethyl pimelimidate-2HCl (DMP) or glutaraldehyde was added to the reaction mixture at a final concentration of 5 m*M* and 0.05%, respectively, and incubated for an additional 15 min at room temperature. Crosslinking was terminated by bringing the solution to 100 m*M* Tris–HCl, pH 7.5, and the products were then analyzed by 10% SDS–PAGE and visualized by immunoblotting.

RESULTS

Expression and purification of NF-κB, IRF-1, and Tax proteins

To obtain relatively large amounts of protein for *in vitro* studies, the *E. coli* glutathione *S*-transferase gene fusion and baculovirus expression systems were used to express NF-κB subunits (Fig. 1A). The cDNAs encoding the NF-κB subunits p50, p52, and pN′65 were expressed as glutathione *S*-transferase fusion proteins and purified from bacterial lysates by affinity chromatography using glutathione–Sepharose 4B. The c-Rel, IRF-1, and Tax proteins were separately expressed in baculovirus as polyhistidine-tagged proteins and isolated from insect cell lysates under native elution conditions by a rapid affinity purification using His-Bind metal chelation resin. Purified proteins were analyzed by SDS–polyacrylamide gel electrophoresis; the purity estimated from Coomassie stained gels was 60, 90, 80, 90, 70, 80, and 90% for c-Rel, Tax, N′p65, N′IRF-1, IκBα, p52, and p50, respectively (Fig. 1B; lanes 1–7, respectively).

Tax stimulates NF-κB binding to the NF-κB DNA binding sequence

Tax enhances binding and dimerization of the CREB/ATF family of bZIP proteins (Wagner *et al.,* 1993; Giovanni *et al.,* 1995). To determine whether Tax similarly enhanced binding of NF-κB to DNA, recombinant NF-κB proteins were tested for their ability to bind to DNA in the presence or absence of Tax in EMSA (Fig. 2), using the HIV-1 NF-κB or MHC-I probes and the PRD1 probe for IRF-1 binding. NF-κB proteins were incubated without Tax (Fig. 2, lanes 1, 4, 7, and 10), with Tax (Fig. 2, lanes

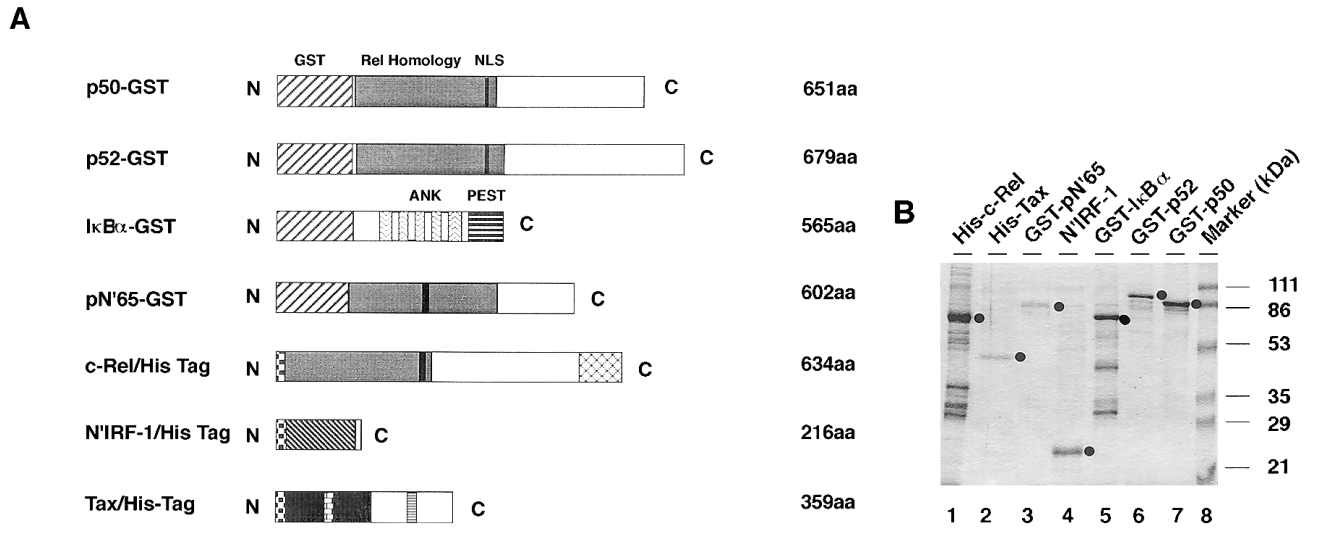


FIG. 1. Expression of recombinant proteins. (A) Schematic representation of recombinant NF-κB proteins expressed as glutathione *S*-transferase (GST) fusion proteins or as polyhistidine-tagged proteins. The histidine-tagged proteins contain 12–21 histidine residues at their N-termini (▩). The boxes represent different protein domains: ■, Rel homology domain; ▨, transactivation domain; ▤, ankyrin repeat; ▤, PEST domain; ▨, glutathione *S*-transferase; ▧, IRF-1 DNA-binding domain; ■, nuclear localization sequences; ▤, CREB binding site in Tax; ▩, sequence required for NF-κB activation in Tax. (B) Coomassie-stained SDS–polyacrylamide gel of purified recombinant proteins (100 ng each); the dots indicate the full-length protein.

2, 5, 8, and 11), or with baculovirus Sf9 extract (Fig. 2, lanes 3, 6, 9, and 12) for 60 min. Tax differentially stimulated binding of all NF-κB subunits to DNA 15-, 25-, 30-, and 30-fold for p52, p50, c-Rel, and p65, respectively (Fig. 2; lanes 2, 5, 8, and 12). The electrophoretic mobility of the NF-κB/DNA complexes was not altered by the presence of Tax, indicating that Tax affected NF-κB DNA binding without forming a ternary complex with NF-κB and DNA. Tax had no intrinsic DNA-binding activity (Fig. 2, lane 13) and the effect of Tax was specific since enhanced binding was not observed using Sf9 cell extract alone (Fig. 2, lanes 3, 6, 9, and 12); similarly Sf9 extract alone displayed no intrinsic NF-κB-like binding activity (Fig. 2, lane 14).

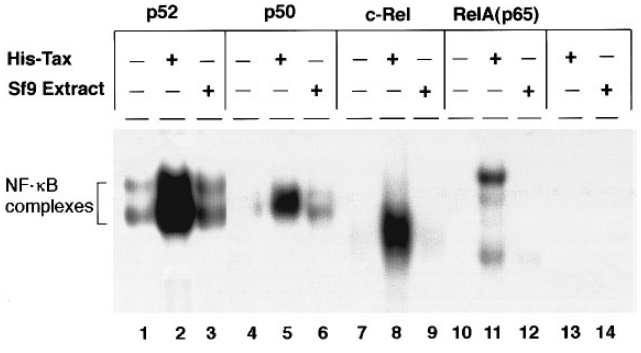


FIG. 2. Enhanced NF-κB-DNA binding mediated by Tax. Recombinant proteins of p52 (0.5 ng), p50 (0.2 ng), c-Rel (0.2 ng), and p65 (1.0 ng) were assayed for binding to 0.2 ng of MHCI probe (for p52; lanes 1–3) or to 0.2 ng of HIV-1 enhancer probe (lanes 4–12), in the absence of Tax (lanes 1, 4, 7, and 10), in the presence of Tax (25 ng) (lanes 2, 4, 8, and 11), or in the presence of Sf9 extract (25 ng) (lanes 3, 6, and 9). Lanes 13 and 14 contain His-Tax and Sf9 extract (25 ng each), respectively.

Association of NF-κB/Rel with DNA is increased by Tax

To investigate the association of NF-κB with DNA, binding of NF-κB to DNA at different times after Tax addition was measured (Fig. 3). In the absence of Tax, only weak binding of c-Rel (0.2 ng), p65 (1.0 ng), and p52 (0.5 ng) was observed even after 60 min of incubation with radiolabeled probe (Fig. 3A, lane 7; Fig. 3B, lane 1; and Fig. 3C, lane 1, respectively). In the presence of Tax, the level of NF-κB increased dramatically, reaching a maximum at 20 to 30 min for c-Rel (Fig. 3A; lanes 2–4), p65 (Fig. 3B; lanes 2–5), and p52 (Fig. 3C; lanes 7 and 8). The total NF-κB protein–DNA binding in the presence of Tax increased approximately 30-, 35-, and 20-fold for p65, c-Rel, and p52, respectively. In contrast, recombinant IRF-1 protein was not affected by incubation with Tax (Fig. 3A; lanes 9–11) (Wagner *et al.,* 1993). This result indicates that Tax can increase the on-rate of NF-κB–DNA-binding activity. To determine the effect of Tax on the dissociation of NF-κB–DNA complexes, a reaction mixture of p65–DNA was incubated in the absence or presence of Tax for 40 min, followed by the addition of a 100-fold excess of specific competitor DNA (Fig. 4). Note that in order to obtain approximately equivalent amounts of p65–DNA complex, a 30-fold excess of p65 was added to the reaction mixture without Tax (Fig. 4, lanes 5–7). The amount of NF-κB–DNA complex remaining after addition of DNA, measured as a function

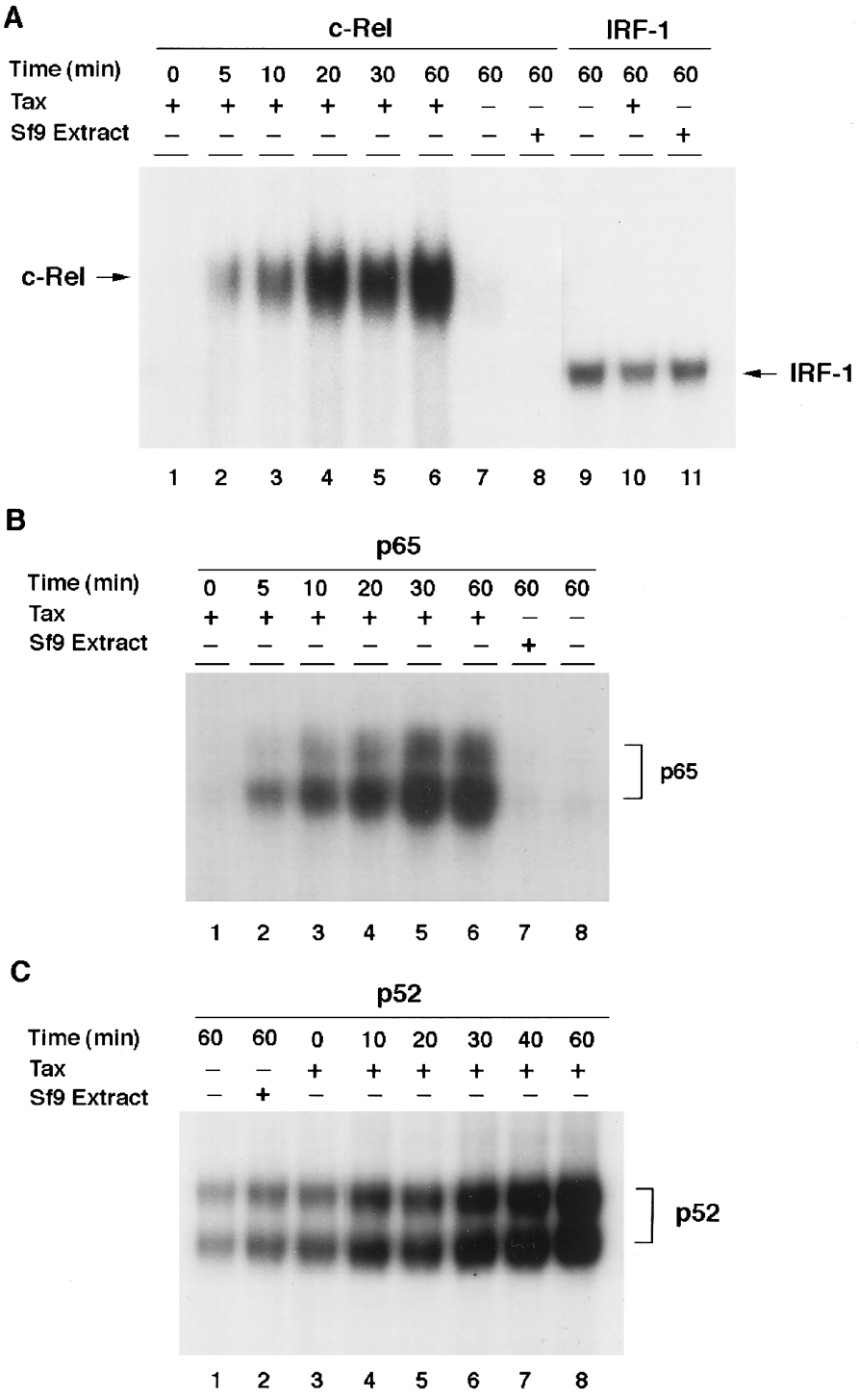


FIG. 3. Association of NF-κB/Rel with DNA is mediated by Tax. (A) Recombinant c-Rel (0.2 ng) was incubated with 0.2 ng of HIV-1 enhancer probe and Tax (25 ng) for 0–60 min (lanes 1–6). c-Rel was incubated with probe for 60 min alone (lane 7) or with Sf9 extract (lane 8). IRF-1 (0.2 ng) was incubated alone (lane 9) with Tax (25 ng) (lane 10) or with Sf9 extract (25 ng) (lane 11) for 60 min. (B) p65 (1.0 ng) was incubated with Tax (25 ng) for 0–60 min (lanes 1–6) or without Tax for 60 min (lanes 7 and 8). (C) NF-κB2(p52) (0.5 ng) was incubated without Tax for 60 min (lanes 1 and 2) or with Tax (25 ng) for 0–60 min (lanes 3–8).

of time, was analyzed by EMSA. As shown in Fig. 4, p65 dissociated from the DNA probe at a comparable rate in the presence (Fig. 4; lanes 2–4) or absence of Tax (Fig. 4; lanes 5–7); similar results were also observed for other NF-κB subunits (data not shown). Thus, Tax affects the association of NF-κB with the DNA probe but does not alter the dissociation of NF-κB–DNA complexes.

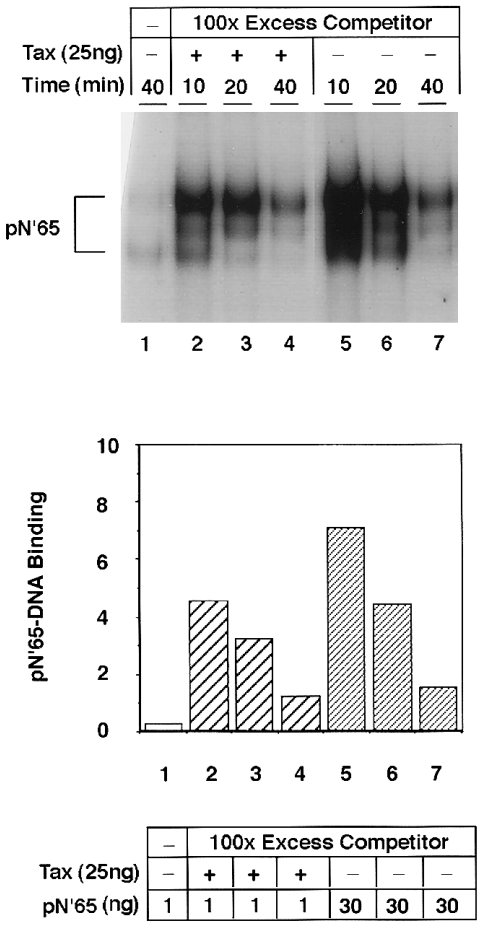


FIG. 4. Dissociation of pN′65–DNA complexes is not affected by Tax. Dissociation of pN′65 from probe DNA was analyzed in the presence of pN′65 (1.0 ng) and Tax (25 ng) (lanes 2–4) or with pN′65 (30 ng) and in the absence of Tax (lanes 5–7). The complex was allowed to form for 40 min, followed by addition of a 100-fold excess of unlabeled HIV-1 probe for 10, 20, or 40 min (lanes 2–7). In lane 1, the amount of pN′65 (1.0 ng) binding at 40 min in the absence of Tax is shown.

Tax increases the formation of NF-κB/Rel dimers in the absence of DNA

NF-κB/Rel proteins contact DNA as dimers and dimerization is a prerequisite for DNA binding (Grimm *et al.,* 1993; Roulston *et al.,* 1995). Tax may therefore increase protein dimerization or the subsequent interaction between the NF-κB/Rel dimer and DNA. To determine if Tax was involved in the enhancement of dimerization, a protein–protein chemical crosslinking analysis was performed. GST-p52 was incubated in the presence or absence of Tax and after addition of the chemical crosslinker DMP, the products were fractionated on an SDS–PAGE gel and analyzed by immunoblotting (Fig. 5A). GST-p52 at a concentration of 50 ng migrated predominantly as a monomer of 82 kDa (Fig. 5A; lane 6); increasing the concentration of GST-p52 to 100 ng resulted in a small amount of dimerization, with some crosslinked protein at 164 kDa (Fig. 5A; lane 7). The addition of increasing concentrations of Tax (50–200 ng) increased the formation of homodimers by 40- to 110-fold (Fig. 5A; lanes 3–5); nonspecific formation of a 164-kDa complex did not occur in the absence of DMP (Fig. 5A; lanes 1 and 2). This protein–protein crosslinking experiment illustrates that Tax alone in the absence of DNA increases the formation of NF-κB2 p52 dimers. Immunoblots were stripped and probed with anti-Tax antibody; no Tax was present in the 82- or 164-kDa bands, indicating that Tax was not a component of the observed complex (data not shown). Similarly, Tax also enhanced the dimerization of p65 (Fig. 5B, lanes 2 and 3); dimerization of p65 did not occur to an appreciable extent in the absence of Tax (Fig. 5B, lanes 1 and 5).

Effect of Tax on NF-κB binding activity in the presence of IκBα

NF-κB–DNA complex formation can be inhibited *in vitro* by incubation with IκBα; in addition, IκBα is able to dissociate preformed protein–DNA complexes (Urban *et al.,* 1990). This property of IκBα may be relevant to the inhibition of NF-κB mediated activation of gene expression since recent studies have demonstrated that newly synthesized IκBα can be localized to the nucleus (Arenzana-Seisdedos *et al.,* 1995). To examine the effect of IκBα on Tax-stimulated NF-κB binding activity, the binding of p65 to NF-κB DNA in the presence of Tax and/or IκBα was examined. As shown in Fig. 6, p65 binding to the NF-κB element was stimulated in the presence of Tax (Fig. 6; lane 2) but was inhibited by the addition of IκBα (Fig. 6; lane 4). Addition of increasing amounts of IκBα (1, 5, and 10 ng) with p65, followed by the addition of a constant amount of Tax (Fig. 6; lanes 5–7), resulted in complete inhibition of p65–DNA complex formation at the higher IκBα concentrations. However, in the reverse reaction with increasing concentrations of Tax and a constant amount of IκBα, p65 DNA binding was stimulated (Fig. 6, lanes 8–12), illustrating that Tax was able to overcome the inhibitory effect of IκBα. In previous experiments, we also observed that Tax could overcome the inhibitory effects of IκBα on gene expression (Lacoste *et al.,* 1994), thus supporting the idea that Tax and IκBα have antagonistic effects on NF-κB binding.

Tax directly associates with the inhibitor IκBα

Next, *in vitro* GST-affinity chromatography was used to determine whether Tax can associate with IκBα directly, as well as with other recombinant NF-κB proteins (Fig. 7). Equivalent amounts of GST–NF-κB subunits and GST–IκBα were immobilized on GST–Sepharose beads followed by the addition of His-Tax. As expected, Tax interacted with each of the NF-κB subunits p50, p52, p65, and c-Rel (Fig. 7; lanes 1, 3, 5, and 6) but not with GST alone; a strong Tax–IκBα interaction was also observed (Fig. 7; lane 4). To determine if the Tax–IκBα interaction

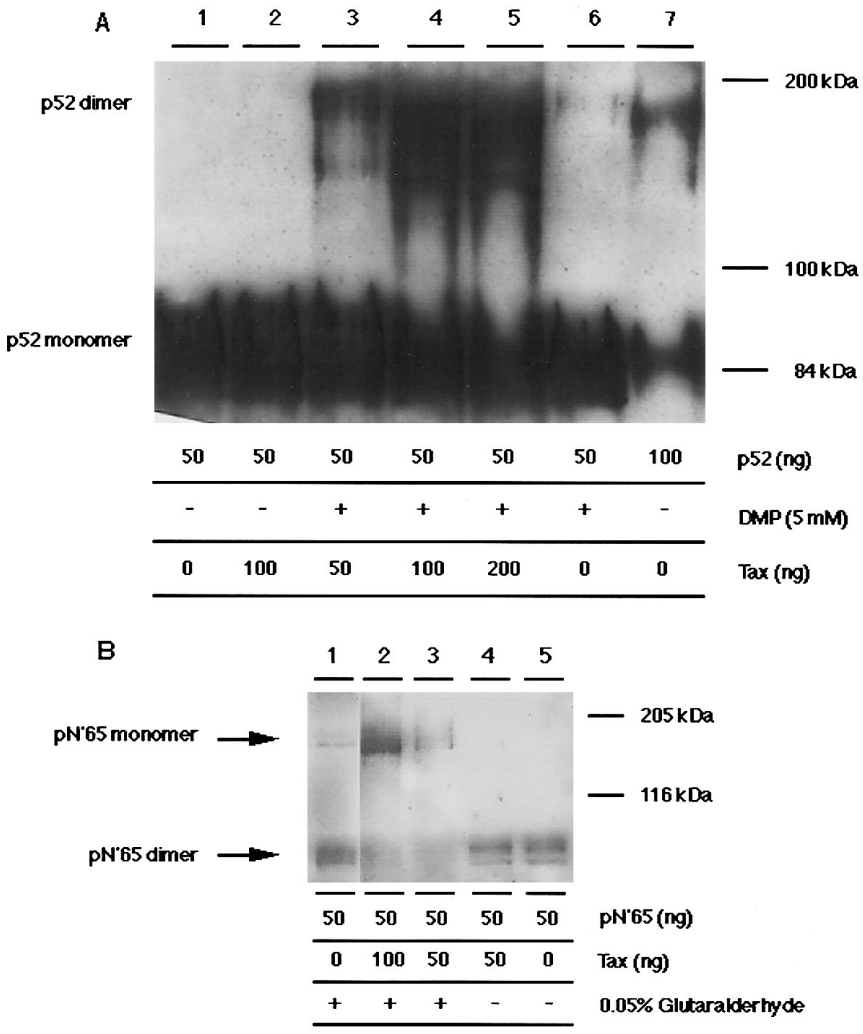


FIG. 5. Tax mediated enhancement of dimerization of NF-κB2(p52) and pN′65. A Recombinant GST-p52 was incubated in the presence (lanes 2–5) or absence of Tax (lanes 1, 6, and 7). Reaction mixtures were treated with 5 m*M* DMP crosslinker (lanes 3–7) in the presence of GST-p52 and Tax at 50–200 ng (lanes 3–5) or GST-p52 alone at concentrations of 50 and 100 ng, respectively (lanes 6 and 7). (B) GST-pN′65 was crosslinked using 0.05% glutaraldehyde (lanes 1–3). pN′65 at 50 ng was incubated alone in the presence of crosslinker (lane 1). Reaction mixtures were incubated with pN′65 (50 ng) and with 100 or 50 ng of Tax (lanes 2 and 3). Lanes 4 and 5 contain reactions of pN′65 with Tax and pN′65 in the absence of crosslinker, respectively. The products were fractionated by 10% SDS–PAGE and detected by immunoblotting.

observed *in vitro* was also observed *in vivo,* N-Tera 2 cells were cotransfected with plasmids expressing Tax and the truncated form of IκBα, IκBΔ4 (a C-terminal deletion of aa296-317) (Lin *et al.,* 1996; Beauparlant *et al.,* 1996c). As illustrated in Fig. 8, immunoprecipitation with anti-Tax antibody from transfected cells also immunoprecipitated endogenous IκBα and increasing amounts of IκBαΔ4 (Fig. 8B, lanes 1–4). Similarly, the Tax mutants M22 (NF-κB$^-$/CREB$^+$) and M47 (NF-κB$^+$/CREB$^-$) (Smith *et al.,* 1990) also immunoprecipitated endogenous IκBα and increasing amounts of transfected IκBαΔ4 (Fig. 8B; lanes 5–9). The reverse co-immunoprecipitation was also performed and anti-IκBα antibody immunoprecipitated endogenous IκBα, increasing amounts of transfected IκBαΔ4 and wild-type Tax (Fig. 8A; lanes 1–4), mutant Tax M22 (Fig. 8A; lanes 5–8), and Tax M47 (Fig. 8A; lane 9).

Co-immunoprecipitation studies were also performed using the HTLV-1 transformed T cell line C8166, which expresses relatively high levels of Tax, displays constitutive NF-κB binding activity, and has a higher rate of IκBα turnover compared to nontransformed T cell lines (Kanno *et al.,* 1994a; Sun *et al.,* 1994; Lacoste *et al.,* 1995). Surprisingly, with the immunoprecipitation conditions used in the previous experiment, Tax was unable to co-immunoprecipitate IκBα (Fig. 8C; lane 4), possibly due to the high rate of IκBα turnover (Suzuki *et al.,* 1995). To circumvent this possibility, calpain inhibitor I and the antioxidant pyrrolidine dithiocarbamate (PDTC) were used to block IκBα degradation (Beauparlant *et al.,* 1996a). PDTC is both a metal chelator and an antioxidant that absorbs metal ions required for the Haber Weiss reaction (Schreck *et al.,* 1991) while calpain inhibitor I inhibits proteasome mediated catalytic activity (Ciechanover,

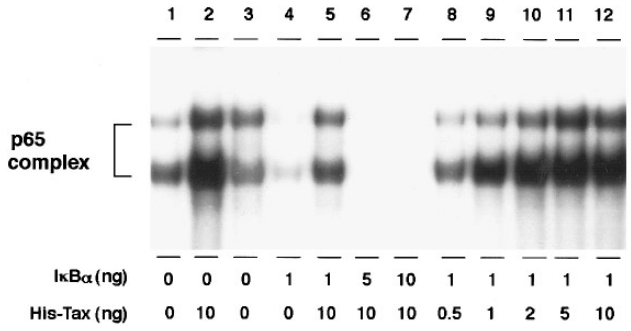


FIG. 6. Competition for NF-κB binding activity by Tax and IκBα. Recombinant p65 (1.0 ng) was incubated with Tax (10 ng) and increasing concentrations of IκBα (1–10 ng) (lanes 5–7), with IκBα added to the reaction mixture before Tax. IκBα (1.0 ng) was incubated with increasing concentrations of Tax (0.5–10 ng) (lanes 8–12). Tax was added to the reaction mixture with IκBα (lanes 5–12); p65 protein was incubated with HIV-1 enhancer probe alone (lane 1) with Tax (lane 2) with Sf9 extract (lane 3) and with IκBα (lane 4).

1994). Both PDTC and calpain inhibitor I block NF-κB activation by interfering with IκBα degradation (Sun *et al.,* 1993; Beg *et al.,* 1993b; Henkel *et al.,* 1993; Traenckner *et al.,* 1994; Beauparlant *et al.,* 1996b); PDTC stabilizes IκBα by blocking its phosphorylation whereas calpain inhibitor permits the accumulation of the phosphorylated and ubiquitinated form of IκBα (Beauparlant *et al.,* 1996b) but blocks at the level of proteasome mediated degradation. Interestingly, in C8166 cells treated with calpain inhibitor I, IκBα was not immunoprecipitable with an anti-Tax antibody (Fig. 8C, lanes 4 and 6). However, from PDTC treated cells, Tax and IκBα were co-immunoprecipitated (Fig. 8; lane 5), suggesting that Tax may preferentially interact with an unmodified form of IκBα.

To further characterize the antagonistic effects of Tax and IκBα on regulation of NF-κB dependent gene expression, a series of cotransfection experiments was performed in N-Tera-2 cells using the HIV-1 LTR-CAT reporter gene (Δ23/CAT) and different amounts of Tax, IκBα, and p65 expression plasmids. Cotransfection of p65, Tax, or Tax and p65 expression plasmids increased the activity of the HIV-1 LTR at 48 hr after transfection between 5- and 20-fold (Fig. 9, lanes 2–4), while the addition of IκBα together with p65 eliminated gene activity (Fig. 9, lane 5). The addition of increasing amounts of Tax plasmid was able to reverse the inhibitory effects of IκBα on gene activity Fig. 9, lanes 6–9), indicating that the Tax–IκBα antagonistic effects observed in DNA-binding analysis are also reflected in changes in NF-κB dependent gene activity.

DISCUSSION

The human T cell leukemia virus type 1 Tax protein activates viral and cellular gene transcription indirectly by interacting with transcription factors such as CREB/ATF, p67^{SRF}, Ets-1, and NF-κB family members (reviewed in Hiscott *et al.,* 1995). Physical association of Tax with the CREB/ATF family was shown to increase protein dimerization and subsequent binding of the dimer to DNA (Suzuki *et al.,* 1993; Goren *et al.,* 1995; Kwok *et al.,* 1996; Bantignies *et al.,* 1996). *In vivo* studies in HTLV-1 infected and Tax expressing cells also displayed enhanced NF-κB binding activity by EMSA, as well as increased degradation of IκBα turnover by immunoblot analysis (Kanno *et al.,* 1994a; Sun *et al.,* 1994; Lacoste *et al.,* 1995; Brockman *et al.,* 1995). In fact, immunoprecipitation studies have demonstrated that Tax interacted predominantly with NF-κB2 (p100/p52), as well as with NF-κB1 (p105/p50), p65, and c-Rel (Hirai *et al.,* 1992; Beraud *et al.,* 1994; Lanoix *et al.,* 1994; Suzuki *et al.,* 1994; Murakami *et al.,* 1995).

In this study we utilized recombinant NF-κB, IκBα, and Tax proteins to evaluate the effects of Tax–NF-κB interactions on DNA binding activity and gene activity. First, we found that Tax specifically stimulated the DNA binding of NF-κB subunits by 20- to 40-fold and the effect was maximal at low concentrations of NF-κB protein. In contrast, Tax mediated enhancement of DNA binding was not observed with the unrelated IRF-1 factor, indicating a specificity of Tax for NF-κB/Rel proteins, as well as bZIP proteins (Wagner *et al.,* 1993). Second, Tax enhanced the amount and the rate of association of NF-κB binding to DNA but had no effect on the rate of dissociation of protein–DNA complexes. Since Tax did not affect the rate of NF-κB dissociation and has not been shown to be part of the NF-κB/DNA complex, it appears that Tax binds to NF-κB weakly, but is nonetheless sufficient to stimulate dimerization. Once dimerization has occurred, Tax may no longer be required in the NF-κB/DNA complex. This model, as originally proposed for Tax–bZIP interactions, could explain why Tax is not observed as a component of the NF-κB/DNA complex (Wagner *et al.,* 1993; Giovanni *et al.,* 1995).

Another point at which Tax may act upon the NF-κB pathway is at the level of regulation by IκB. Several re-

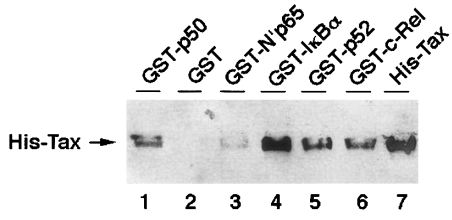


FIG. 7. *In vitro* binding of Tax protein to different NF-κB proteins. GST-NF-κB-p50, p65, p52 (lanes 1, 2, and 4), His-tagged c-Rel (lane 6), GST alone (lane 2), and IκBα (lane 3) proteins (300 ng) were immobilized onto glutathione Sepharose beads or His-Bind metal chelation resin; affinity chromatography using His-Tax (50 ng) was performed as described under Materials and Methods. Lanes 7 and 8 respectively contained 25 and 50 ng of Tax alone. Recombinant Tax protein retained by the fusion proteins was eluted and visualized by immunoblot analysis with anti-Tax antibody.

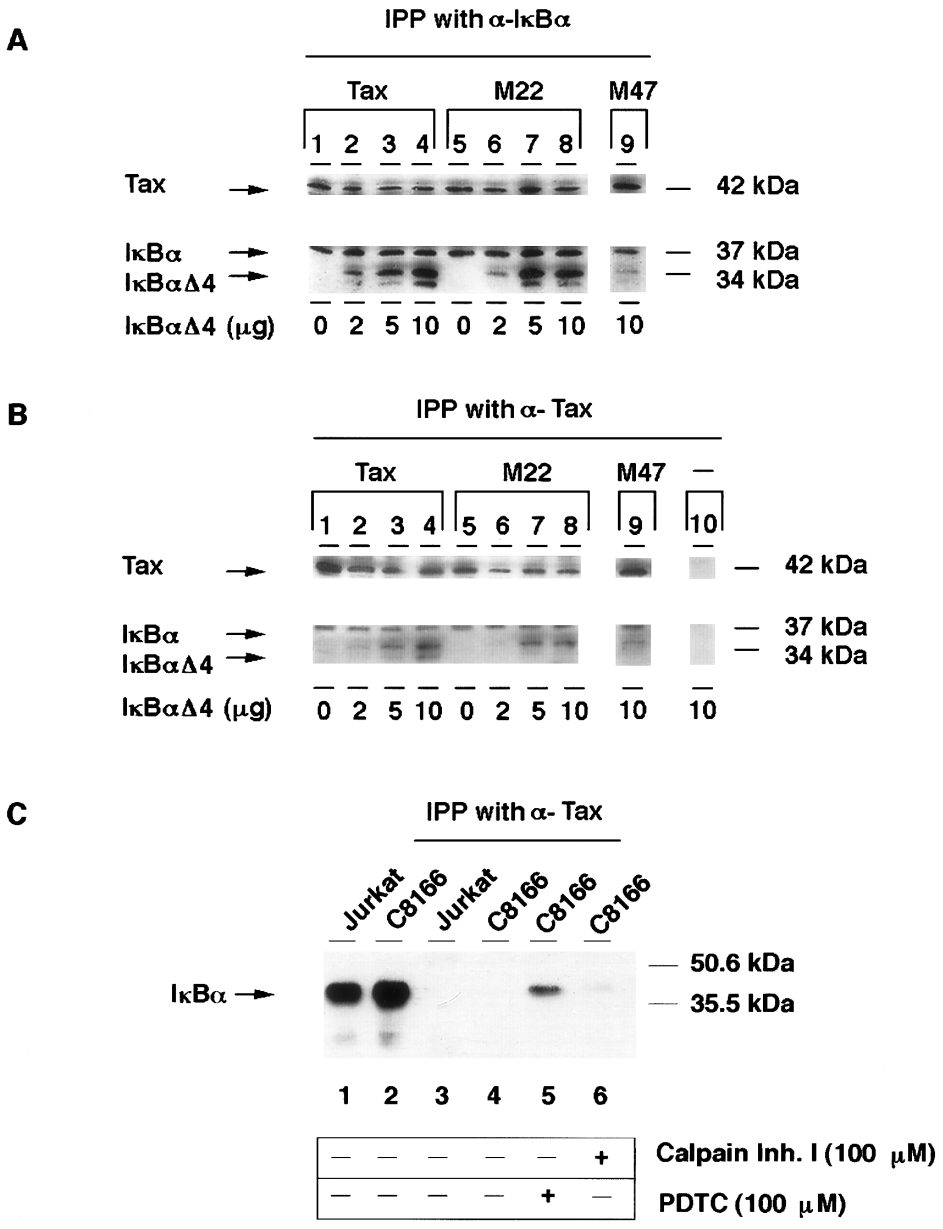
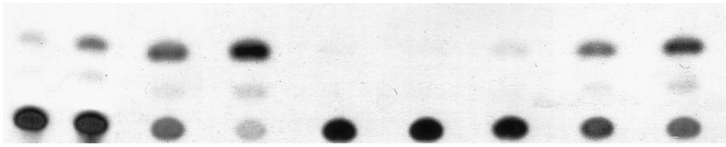


FIG. 8. Co-immunoprecipitation of Tax and IκBα. (A) N-Tera 2 cells were transfected with expression plasmids encoding IκBαΔ4 (0–10 μg) and wild-type Tax (10 μg) (lanes 1–4), mutant Tax M22 (lanes 5–8), or mutant M47 (lane 9). Immunoprecipitation was performed with anti-IκBα antibody and co-immunoprecipitated Tax was detected by immunoblot with anti-Tax antibody. (B) The reverse immunoprecipitation was performed with anti-Tax antibody and blotted with anti-IκBα antibody. (C) Co-immunoprecipitation of Tax and IκBα from C8166 cells. C8166 cells were treated with PDTC (100 μM) (lane 5) or calpain inhibitor I (100 μM) (lane 6) for 1 hr or left untreated (lane 4). Immunoprecipitations were performed using anti-Tax antibody and co-precipitated IκBα was detected by immunoblot with anti-IκBα antibody. Non-Tax-expressing Jurkat cells served as control (lane 3). Lanes 1 and 2 indicate the endogenous levels of IκBα from Jurkat cells and C8166 cells, respectively.

cent studies have examined the requirements for inducer mediated degradation of IκBα. Following inducer mediated stimulation by a variety of activators including HTLV-1 Tax, IκBα becomes hyperphosphorylated, detectable in immunoblots as a slowly migrating form and sensitive to phosphatase treatment (Beg *et al.,* 1993b; Brown *et al.,* 1993). Hyperphosphorylation occurs at the N-terminus of the molecule and is a signal for subsequent ubiquitination and degradation by the 26S proteasome (Alkalay *et al.,* 1995a; Finco *et al.,* 1994; Lin *et al.,* 1995; Traenckner *et al.,* 1994; Chen *et al.,* 1995). Moreover, only hyperphosphorylated IκBα is a target for degradation by an *in vitro* reconstituted ubiquitin–proteasome system (Alkalay *et al.,* 1995b). Phosphorylation and subsequent degradation via the ubiquitin–proteasome degradation pathway are therefore key elements in NF-κB liberation and nuclear translocation (Beg *et al.,* 1993b; Brown *et al.,* 1993; Chen *et al.,* 1995; Alkalay *et al.,* 1995a,b; Finco *et al.,* 1994; Traenckner *et al.,* 1994; Lin *et al.,* 1995). Substitution of serine 32 and/or 36 to alanine abolishes inducer medi-



3	3	3	3	3	3	3	3	3	Δ23/CAT
—	3	—	3	3	3	3	3	3	p65
—	—	10	10	—	1	5	10	10	Tax
—	—	—	—	1	1	1	1	1	IκBα

Calpain Inh. I
125 μM
6Hrs

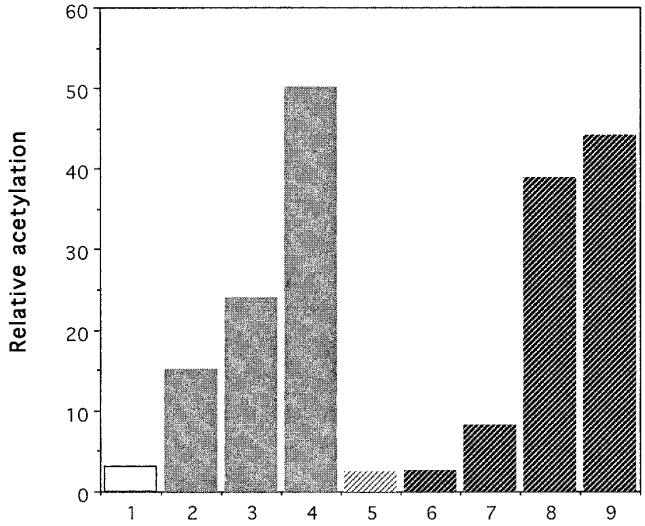


FIG. 9. Analysis of the effect of Tax-IκBα antagonism on gene activity. Human N-Tera-2 cells were transfected by the calcium phosphate method with Δ23/CAT (3 μg) (Δ23/CAT contains the −167 to +80 region of the HIV-1 LTR linked to CAT and includes the two NF-κB binding sites in the −100 region) and with the indicated amounts of the CMV-based p65 and Tax cDNA expression vectors and SVK3-based IκBα expression vector. At 40 hr after transfection, the cells were either treated with Calpain Inhibitor I (125 μ*M*) for 6 hr (lane 8) or not treated (lane 9), the cells were harvested, and protein extracts (300 μg) were subjected to CAT analysis. The percentage acetylation was determined by quantifying the acetylated and substrate chloramphenicol. The results, presented as a bar graph, are representative of three independent experiments.

ated phosphorylation and degradation of IκBα (Brown *et al.,* 1995; Whiteside *et al.,* 1995; Brockman *et al.,* 1995; Chen *et al.,* 1995). These residues thus represent the critical signal response phosphoacceptor sites of multiple activators of NF-κB, including TNF-α, LPS, and HTLV-1 Tax. Likewise mutation of sites of IκBα ubiquitination at Lys-21 and Lys-22 also generates a signal nonresponsive form of IκBα (Scherer *et al.,* 1995). Interestingly, a 700-kDa complex involved in the phosphorylation of IκBα at Ser-32/Ser-36 has been identified, that itself requires ubiquitination for activation of the kinase activity (Chen *et al.,* 1996). IκBα is also constitutively phosphorylated at multiple sites in the carboxyl-terminal PEST domain by casein kinase II (CKII); phosphorylation at these sites is responsible for the short half-life of the protein (McElhinny *et al.,* 1996; Barroga *et al.,* 1995; Lin *et al.,* 1996); mutation of the CKII sites in the C-terminal domain of IκBα results in a protein with increased intrinsic stability (Lin *et al.,* 1996).

Tax has been shown to indirectly activate NF-κB activity by enhancing IκBα degradation (Lacoste *et al.,* 1995; Sun *et al.,* 1994; Kanno *et al.,* 1994b); furthermore mutation of Ser-32 and/or Ser-36 prevents Tax mediated degradation of IκBα (Brockman *et al.,* 1995; Kanno *et al.,* 1995). Tax also physically interacts with IκBα via the ankyrin repeats (Hirai *et al.,* 1994). We provide additional evidence that Tax can either compete for or physically remove IκBα from the NF-κB–DNA complex, since Tax can overcome IκBα mediated inhibition of NF-κB binding in EMSA. The recent observation that IκBα can shuttle into the nucleus (Arenzana-Seisdedos *et al.,* 1995) also lends support to the idea that IκBα may directly inhibit

gene expression by dissociating NF-κB–DNA complexes *in vivo.* In support of Tax–IκBα interactions, binding of Tax to the NF-κB1 and NF-κB2 molecules via the ankyrin repeats was shown previously to increase nuclear translocation of Rel/NF-κB proteins (Lanoix *et al.,* 1994; Sun *et al.,* 1994; Beraud *et al.,* 1994; Kanno *et al.,* 1994b). Other studies using transient transfection in NF-κB mediated gene expression assays demonstrated that increasing amounts of Tax resulted in the stimulation of NF-κB mediated gene activity despite the presence of IκBα (Lacoste *et al.,* 1994; Maggirwar *et al.,* 1995), although the dose-dependent effect of Tax was abolished when cells were pretreated with inhibitors of IκBα degradation (Maggirwar *et al.,* 1995). Using affinity chromatography, transient transfection and immunoprecipitations, we were able to demonstrate Tax–IκBα interactions. Interestingly, wild-type Tax as well as mutant Tax M22 (NF-κB$^-$/CREB$^+$) and M47 (NF-κB$^+$/CREB$^-$) were able to bind to IκBα *in vivo.* The fact that the Tax M22 mutant was still able to bind to IκBα, despite a block in NF-κB mediated transactivation (Smith *et al.,* 1990), suggests that the inability of Tax M22 to activate NF-κB function may not be due to a block in Tax–NF-κB/IκB interaction but rather to an inability of Tax M22 to target IκBα for degradation.

In this regard, Tax was found recently to associate with two subunits of the 20S proteasome, HsN3 and HC9 through binding to NF-κB1(p105) (Rousset *et al.,* 1996). Tax–NF-κB1(p105) association led to increased proteasome mediated processing of the p105 precursor to the DNA binding form of p50 (Rousset *et al.,* 1996). These experiments provide a vital link between the proteasome and NF-κB on the one hand and Tax activity on the other. However, the increased proteolysis of NF-κB1 in the presence of Tax is limited (Rousset *et al.,* 1996) and also does not account for the dramatic increases in NF-κB2 and c-Rel levels observed in HTLV-1 transformed and Tax-expressing cell lines (Lanoix *et al.,* 1994; Beraud *et al.,* 1994; Kanno *et al.,* 1994a; Suzuki *et al.,* 1995; Crenon *et al.,* 1993). Nevertheless, Tax may likewise target IκBα for degradation via the same mechanism. The inability of Tax M22 to mediate NF-κB induction may be due to the inability of Tax M22 to bind to the proteasome and form a ternary complex. Interestingly, our preliminary evidence indicates that Tax may bind preferentially to unmodified IκBα, since it was not possible to immunoprecipitate a Tax–IκBα complex from C8166 cells after treatment with calpain inhibitor I, which blocks the activity of the proteasome but not IκBα phosphorylation or ubiquitination. Compounds such as calpain inhibitor I and MG132 would thus be expected to abolish Tax mediated NF-κB transactivation (Maggirwar *et al.,* 1995). On the other hand, the use of PDTC, which effectively blocks IκBα phosphorylation, permitted the immunoprecipitation of Tax–IκBα complexes. Further studies are required to establish whether Tax can differentially recognize the unmodified versus modified forms of IκBα. The ability of Tax to stimulate NF-κB binding activity thus occurs at multiple levels (Hiscott *et al.,* 1995) including Tax enhanced dimerization of NF-κB subunits in the absence of DNA, stimulation of NF-κB binding, physical interaction of Tax with IκBα, and interference with the regulatory function of IκBα. Ultimately, Tax may also target IκBα for degradation by acting as a viral chaperonin to direct IκBα to the proteasome.

ACKNOWLEDGMENTS

These studies were supported by a grant from the National Cancer Institute of Canada. We thank Dr. R. T. Hay for the kind gift of monoclonal IκB antibody MAD10B and Dr. W. C. Greene for the Tax mutants. We also thank the AIDS Research and Reference Reagent Program for the anti-Tax antibody and C8166 cells. J.H. is the recipient of a MRC Scientist Award.

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