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Inactivation of IκBβ by the Tax Protein of Human T-Cell Leukemia Virus Type 1: a Potential Mechanism for Constitutive Induction of NF-κB

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In resting T lymphocytes, the transcription factor NF-κB is sequestered in the cytoplasm via interactions with members of the IκB family of inhibitors, including IκBα and IκBβ. During normal T-cell activation, IκBα is rapidly phosphorylated, ubiquitinated, and degraded by the 26S proteasome, thus permitting the release of functional NF-κB. In contrast to its transient pattern of nuclear induction during an immune response, NF-κB is constitutively activated in cells expressing the Tax transforming protein of human T-cell leukemia virus type 1 (HTLV-1). Recent studies indicate that HTLV-1 Tax targets IκBα to the ubiquitin-proteasome pathway. However, it remains unclear how this viral protein induces a persistent rather than transient NF-κB response. In this report, we provide evidence that in addition to acting on IκBα, Tax stimulates the turnover of IκBβ via a related targeting mechanism. Like IκBα, Tax-mediated breakdown of IκBβ in transfected T lymphocytes is blocked either by cell-permeable proteasome inhibitors or by mutation of IκBβ at two serine residues present within its N-terminal region. Despite the dual specificity of HTLV-1 Tax for IκBα and IκBβ at the protein level, Tax selectively stimulates NF-κB-directed transcription of the IκBα gene. Consequently, IκBβ protein expression is chronically downregulated in HTLV-1-infected T lymphocytes. These findings with IκBβ provide a potential mechanism for the constitutive activation of NF-κB in Tax-expressing cells.

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia, an aggressive and often fatal malignancy of activated $CD4^+$ T lymphocytes (54, 82). This oncogenic retrovirus encodes a 40-kDa protein, termed Tax, which appears to play a central role in the process of neoplastic transformation (31, 50, 55, 69, 75). In this regard, Tax has the capacity to constitutively activate members of the NF-κB/Rel family of transcription factors (5, 44, 59). This virus-host interplay confers Tax inducibility to a set of NF-κB-responsive cellular genes that are normally transcribed at high levels in response to T-cell activation signals. These genes include those encoding the growth factor interleukin-2 (IL-2) and the alpha subunit of its high-affinity receptor (19, 35, 39, 45, 67; for reviews, see references 27 and 28). Recent studies with mice transgenic for the *tax* gene suggest that the constitutive nuclear pattern of NF-κB expression in HTLV-1-infected cells is required to maintain a state of deregulated growth (40). However, the precise mechanism by which Tax induces NF-κB remains unclear.

The prototypic form of NF-κB is a heterodimeric complex containing p50 (NF-κB1) and RelA (p65) (4, 12, 26, 38, 46, 52, 57). In resting T lymphocytes, NF-κB is sequestered in the cytoplasm by virtue of its association with members of a set of inhibitory proteins, including IκBα (2–4, 9, 23). During normal T-cell activation, IκBα is rapidly phosphorylated at Ser-32 and Ser-36 (13, 14, 78), which is a prerequisite for targeting this inhibitor to the ubiquitin-proteasome pathway (16, 62; reviewed in reference 22). Several lines of evidence indicate that Tax stimulates the phosphorylation and degradation of IκBα via a similar mechanism. First, IκBα mutants containing alanine substitutions at Ser-32 and Ser-36 escape from proteolytic breakdown and prevent NF-κB activation in Tax-expressing cells (13). Second, Tax fails to activate latent NF-κB complexes containing mutant forms of IκBα that are defective for ubiquitination (62). Third, point mutations in Tax that ablate its NF-κB-inducing activity also impair its ability to stimulate IκBα turnover (13). Fourth, phosphorylated IκBα accumulates in Tax-expressing cells following treatment with an inhibitor of the proteasome (13). Together, these findings indicate that cytoplasmic NF-κB complexes containing IκBα serve as a major cellular target of HTLV-1 Tax.

Recent molecular cloning studies have revealed another conditionally labile isoform of IκB, termed IκBβ, which has significant sequence homology with IκBα (77). Although IκBα and IκBβ likely interact with the same set of NF-κB/Rel family members, it appears that these two inhibitors display distinct responses to different inducers of NF-κB activity (77). As previously reported (77), agonists that stimulate a transient pattern of NF-κB activity promote the selective breakdown of IκBα. In contrast, agents that elicit a persistent NF-κB response, including bacterial lipopolysaccharide (LPS) and IL-1, are associated with IκBβ inactivation as well (77). However, the relationship between IκBβ, Tax, and the constitutive pattern of NF-κB activation observed in HTLV-1-infected T lymphocytes remains undefined.

In this report, we identify IκBβ as a second proteolytic target of HTLV-1 Tax. As with IκBα (13), Tax stimulates proteasome-dependent turnover of IκBβ by a mechanism that requires the presence of two vicinal serines in its N-terminal region. However, in contrast to IκBα, which is transcriptionally upregulated by Tax, steady-state levels of the IκBβ transcript are not elevated in either Tax-transfected or HTLV-1-infected

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T lymphocytes. Consistent with this observation, we show that the sustained nuclear expression of NF-κB in HTLV-1-transformed cells is associated with chronic downregulation of the IκBβ protein. As such, the capacity of Tax to permanently inactivate IκBβ may reduce the concentration of total intracellular IκB below a threshold level required to render NF-κB exclusively cytoplasmic.

MATERIALS AND METHODS

Plasmids. Complementary DNAs encoding wild-type and mutant forms of HTLV-1 Tax (68), human IκBα (29), and human RelA (57) were cloned into the polylinker of the eukaryotic expression vector pCMV4 (1) downstream of the cytomegalovirus immediate-early promoter. A full-length cDNA encoding IκBβ was isolated by reverse transcription-PCR (24). First-strand DNA was synthesized from 0.1 μg of mouse testis poly(A)$^+$ RNA (Clontech), using Moloney murine leukemia virus reverse transcriptase (Promega) and an oligonucleotide primer designed to hybridize to 3′ noncoding sequences in the IκBβ transcript (5′-CAGGTCTGCAATTTTATTAAGTTGG-3′) (77). The reverse transcription product was amplified by PCR using specific oligonucleotide primers (5′-CCCGGTACCACCATGGCCGGGGTCGCGTGCTT-3′ and 5′-GCTCTAGA GTCAGGCAGGGTTGGGGTCATC-3′) (77). The amplified cDNA was digested with *Kpn*I and *Xba*I, purified by agarose gel electrophoresis, and ligated into the pCMV4 polylinker. Site-directed mutations were introduced into the full-length IκBβ cDNA by the phosphorothioate method (49) and confirmed by DNA sequencing. Epitope-tagged derivatives of IκBβ were constructed by PCR with a 5′ primer that fused sequences encoding the FLAG epitope (11, 56) in frame with N-terminal coding sequences of IκBβ (5′-GGGGTACCACCATGG ACTACAAAGACGATGACGATAAAATGGCCGGGGTCGCGTGCTT-3′). The chloramphenicol acetyltransferase (CAT) reporter plasmid contained the human immunodeficiency virus type 1 (HIV-1) κB enhancer cassette linked to a heterologous TATA box (71).

Cell culture, transfections, and CAT assays. Jurkat leukemic T cells and the HTLV-1-infected T-cell lines Hut102 (25), C8166 (60), MT-2 (48), and MT-4 (47) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. The HTLV-1-infected T-cell line SLB-1 (41) was maintained in Iscove's medium supplemented as described above. Where indicated, cell cultures were treated with the proteasome inhibitor MG132 (100 μM) or lactacystin (20 μM) (both kindly provided by ProScript, Inc.). In some experiments, cells were exposed to the protein synthesis inhibitor cycloheximide (CHX; 50 μg/ml; Sigma). Transfections were performed by either the DEAE-dextran method (34) or electroporation (13). Input DNA was normalized for all transfections by addition of blank pCMV4 vector. For CAT assays, whole cell extracts were prepared and analyzed by the diffusion-based liquid scintillation counting method (51).

Immunoprecipitation and immunoblotting. Cytosolic extracts were prepared as previously described (66) except that the lysis buffer was supplemented with an extensive cocktail of protease inhibitors (7) and phosphatase inhibitors (50 mM sodium fluoride, 10 mM sodium PP_i, 1 μM zinc chloride, 100 μM sodium orthovanadate, and 20 mM β-glycerol phosphate). For purification of epitope-tagged IκBβ, lysates were equilibrated with ELB buffer (13) and fractionated by affinity chromatography with anti-FLAG M2 antibody conjugated to agarose beads (IBI-Kodak). Immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Dupont). Membranes were blocked (13) and immunoblotted with a peptide-specific antiserum for either IκBβ (Santa Cruz Biotechnology; amino acids 330 to 359), IκBα (amino acids 289 to 317), or RelA (amino acids 529 to 551). Some immunoblotting studies were performed with an antiserum prepared against full-length murine IκBβ, which cross-reacts with the human homolog (77) (kindly provided by Sankar Ghosh, Yale University School of Medicine). Immunoreactive proteins were detected by using anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Amersham) in conjunction with the Renaissance chemiluminescence system (Dupont).

Gel retardation assays. Nuclear fractions were prepared from Jurkat T-cell transfectants by high-salt extraction (66) in the presence of protease inhibitors (7). Gel mobility shift assays were performed as previously described (13), using a ^{32}P-radiolabeled palindromic probe derived from κB enhancer sequences in the IL-2 receptor alpha promoter (5′-CAACGGCAGGGGAATTCCCCTCTCC TT-3′) (7).

Northern (RNA) blot analyses. Total RNA was prepared as previously described (18) except that cells were lysed in Tris-buffered 4 M guanidine isothiocyanate (Gibco/BRL) supplemented with 10% Sarkosyl. Poly(A)$^+$ RNA was purified from approximately 750 μg of total RNA by using the polyAtract mRNA isolation system (Promega) and fractionated by electrophoresis on formaldehyde-agarose gels (18). Following electrophoresis, RNA was transferred to Zeta Probe membranes (Bio-Rad) and sequentially hybridized with the indicated ^{32}P-radiolabeled cDNA probes at 42°C in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), 1× Denhardt's reagent, 1% SDS, and 0.1 mg of salmon testis DNA (Sigma) per ml. Radiolabeled probes were generated by using a Random Primed DNA Labeling kit (Boehringer Mannheim).

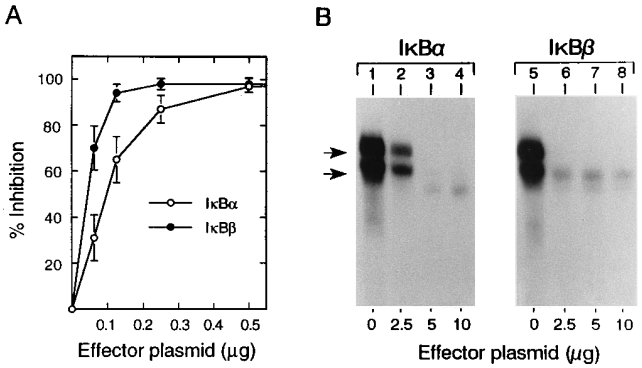

FIG. 1. IκBβ inhibits NF-κB activity in T lymphocytes. (A) Repression of NF-κB-dependent transcription. Human Jurkat T cells were transfected by using DEAE-dextran with a pCMV4-based RelA expression vector (2 μg), graded amounts of the indicated IκB construct, and HIV-κB-CAT (2 μg). After 48 h in culture, whole cell extracts were prepared, normalized for total protein, and assayed for CAT activity. Results from three independent transfections are reported as the mean percent inhibition (± standard error of the mean) of CAT activity in cells expressing IκB effector versus those expressing RelA alone (fold induction = 27.3 ± 2.4). (B) Inhibition of nuclear NF-κB/RelA DNA binding. Jurkat T cells were transfected by electroporation with an expression vector encoding RelA (10 μg) and graded doses of the indicated IκB construct. Approximately 48 h after transfection, cultures were treated with CHX (50 μg/ml) for 2 h. Nuclear protein extracts (5 μg) were added to DNA binding reaction mixtures containing a ^{32}P-labeled palindromic κB probe and analyzed on a 5% native polyacrylamide gel. Nucleoprotein complexes containing RelA homodimers (upper arrow) and RelA/p50 heterodimers (lower arrow) are indicated.

RESULTS

IκBβ regulates NF-κB function in human T lymphocytes. Constitutive nuclear expression of NF-κB in HTLV-1 Tax-expressing cells is associated with proteolytic inactivation of IκBα by the 26S proteasome (13, 62). To establish an in vivo assay for examining whether Tax alters the stability of IκBβ, we first compared the capacities of ectopic IκBα and IκBβ to inhibit NF-κB-dependent transcription in T-cell transfectants. Jurkat T lymphocytes were cotransfected with a CAT reporter plasmid containing two NF-κB binding sites from the HIV-1 enhancer (HIV-κB-CAT), an effector plasmid encoding the RelA transactivating subunit of NF-κB, and graded amounts of expression vectors for either IκBα or IκBβ. As shown in Fig. 1A, both IκBα and IκBβ inhibited RelA-dependent transcription of the CAT reporter gene in a dose-dependent manner. In keeping with these functional results, gel retardation assays performed with nuclear extracts from RelA-expressing transfectants indicated that both IκBα and IκBβ efficiently blocked the DNA binding activity of RelA homodimers (Fig. 1B; upper complex). These inhibitors also sequestered the κB-specific activity of NF-κB (lower complex), which is generated as a consequence of RelA-mediated transactivation of the NF-κB1 gene encoding the p50 subunit (76).

HTLV-1 Tax renders IκBβ susceptible to proteasome-dependent degradation. Having established that ectopic IκBβ inhibits NF-κB in transfected human T cells, we next compared the stabilities of IκBβ and IκBα when independently coexpressed with HTLV-1 Tax. Jurkat T cells were cotransfected with pCMV4 expression vectors (1) encoding FLAG epitope-tagged forms of these inhibitors and wild-type Tax. Transfected cells were exposed to CHX in order to prevent de novo protein synthesis. As shown in Fig. 2A, coexpression with Tax led to a significant reduction in the cytoplasmic levels of both IκBα and IκBβ relative to that observed in Tax-deficient controls. These studies also suggested that IκBβ and IκBα are

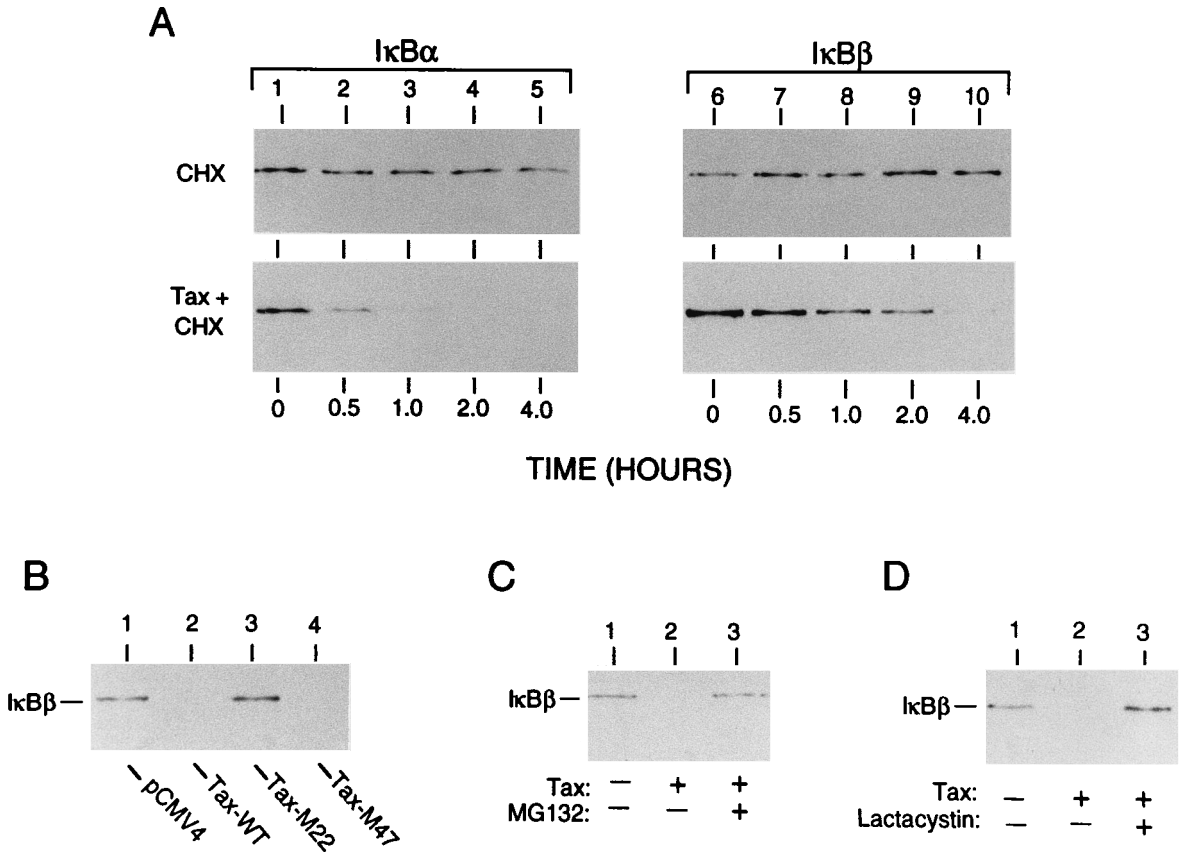

FIG. 2. HTLV-1 Tax stimulates proteasome-dependent degradation of IκBβ. (A) Jurkat T cells were cotransfected by electroporation with expression vectors encoding Tax (10 μg) and either FLAG-tagged IκBα (2 μg) or IκBβ (0.5 μg). Total DNA input was normalized by using the pCMV4 vector. Cytoplasmic extracts were prepared from transfected cells after treatment with CHX for the indicated times. Ectopic forms of IκB were isolated by immunoprecipitation with anti-FLAG antibodies, resolved by SDS-PAGE, and immunoblotted with either IκBα- or IκBβ-specific antiserum. (B) Jurkat cells were cotransfected with expression vectors encoding FLAG-tagged IκBβ (1 μg) and either wild-type Tax (Tax-WT) (10 μg) or a mutant of Tax defective in NF-κB (Tax-M22) or CREB/ATF (Tax-M47) activating function (10 μg). After 48 h in culture, cells were treated with CHX for 6 h. Cytoplasmic extracts were fractionated as described above and immunoblotted with IκBβ-specific antiserum. (C and D) Jurkat T cells were transfected by electroporation with a plasmid encoding FLAG-tagged IκBβ (1 μg) in the absence or presence of a Tax expression vector (10 μg). After 48 h of culture, cells were treated with CHX for 1 h and then exposed to the proteasome inhibitor MG132 (100 μM) or lactacystin (20 μM) for 6 h. Cytoplasmic extracts were fractionated and immunoblotted with an IκBβ-specific antiserum as described above.

degraded with distinct kinetics in Tax-expressing cells. To provide stringent controls for these experiments, we also used vectors for Tax containing missense mutations that selectively disrupt its ability to access either the CREB/ATF (Tax-M47) or NF-κB/Rel (Tax-M22) transcription factor pathway (68). The steady-state level of ectopic IκBβ was substantially reduced in control cells expressing wild-type Tax (Tax-WT) (Fig. 2B, lane 2). Consistent with their differing capacities to induce NF-κB, expression of Tax-M47 but not Tax-M22 resulted in the degradative loss of IκBβ (lanes 3 and 4). These findings correlate the NF-κB-inducing function of Tax with its ability to destabilize IκBβ in transfected T cells.

Recent studies suggest that the constitutive turnover of IκBα in cells expressing Tax is mediated by the ubiquitin-proteasome pathway (13, 62). To examine whether Tax also targets IκBβ to the proteasome, we assessed the ability of the previously described proteasome inhibitors MG132 (53) and lactacystin (21) to block Tax-induced turnover of IκBβ in Jurkat T-cell transfectants. As shown in Fig. 2C, ectopic IκBβ was efficiently degraded in control cells (lane 2) but escaped from Tax-dependent breakdown in cells treated with MG132 (lane 3). Similar results were obtained with lactacystin, a highly specific inhibitor of the proteasome (Fig. 2D). Coupled with the data shown in Fig. 2A and B, these transfection studies implicate the proteasome degradation pathway in the mechanism by which Tax inactivates IκBβ.

Identification of two regulatory serines in the N terminus of IκBβ. IκBα contains two highly conserved serine residues at positions 32 and 36 which are required for its signal-dependent phosphorylation (13, 14, 78), ubiquitination (16, 62), and degradation (13, 14, 16, 78). Disruption of either Ser-32 or Ser-36 in IκBα yields a constitutive NF-κB inhibitor that remains stable despite the presence of HTLV-1 Tax (13). Inspection of the deduced N-terminal sequence of IκBβ (77) revealed a motif containing two serines (Asp-Ser-Gly-Leu-Gly-Ser-Leu; amino acids 18 to 24) that is strikingly similar to that found in IκBα (Asp-Ser-Gly-Leu-Asp-Ser-Met; amino acids 31 to 37) (29). To explore whether Ser-19 and Ser-23 of IκBβ are required for inactivation by Tax, a series of specific amino acid substitutions was introduced into the full-length inhibitor. All of the corresponding mutant proteins were expressed at comparable levels in vivo (data not shown). As illustrated in Fig. 3A, substitution of alanine for either Ser-19 or Ser-23 in ectopic IκBβ (mutants S19A and S23A) markedly attenuated Tax-mediated transactivation of HIV-κB-CAT in Jurkat T-cell transfectants. Similar results were obtained with an IκBβ mutant containing simultaneous Ser→Ala substitutions at these positions (mutant S19/23A). In contrast, IκBβ constructs con-

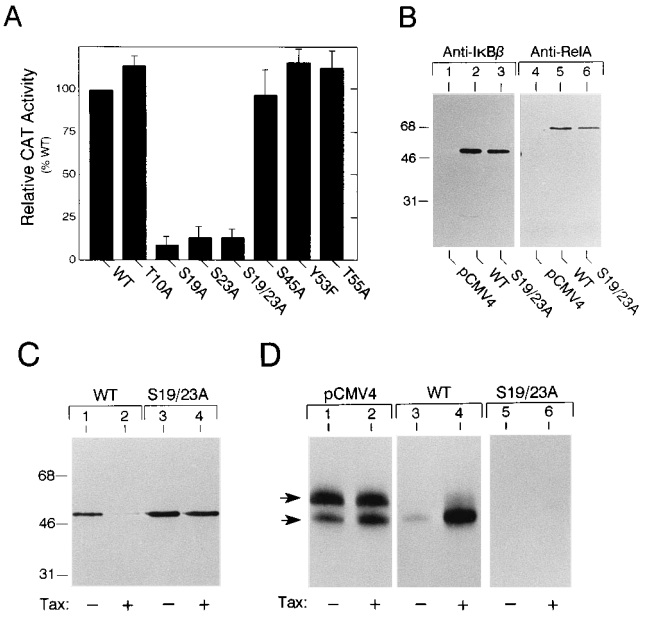

FIG. 3. Serine residues 19 and 23 of IκBβ are required for inactivation by Tax. (A) Induction of NF-κB transcriptional activity. Jurkat T cells were transfected by electroporation with HIV-κB-CAT (5 μg) along with cDNA expression vectors encoding Tax (5 μg) and the indicated site-directed mutants of IκBβ (2.5 μg). After 48 h of growth, cells were harvested and assayed for CAT activity. Tax-dependent increases in reporter gene activity are expressed as a percentage of the activity induced in cells transfected with wild-type IκBβ (WT; fold induction = 32.5 ± 3.4). Each bar represents the mean ± standard error of the mean from three independent experiments. (B) Association with endogenous RelA. Jurkat T cells were transfected by electroporation with expression vectors encoding the indicated FLAG-tagged derivatives of IκBβ (20 μg) or with unmodified pCMV4 vector (20 μg). Cytoplasmic complexes containing ectopic IκBβ were isolated by immunoaffinity chromatography, fractionated by SDS-PAGE, and analyzed by immunoblotting with the indicated antisera. Molecular sizes are given in kilodaltons. (C) Steady-state levels of IκBβ protein. Jurkat T cells were transfected by electroporation with expression plasmids encoding FLAG-tagged wild-type IκBβ (WT; 1 μg) or mutant S19/23A (1 μg) in the absence or presence of Tax-WT (10 μg). After 48 h in culture, cells were treated with CHX for 6 h. Cytoplasmic extracts were prepared, immunoprecipitated with anti-FLAG antibody, and immunoblotted with IκBβ-specific antiserum. Sizes are indicated in kilodaltons. (D) Induction of nuclear NF-κB/Rel activity. Jurkat T cells were transfected by electroporation with expression vectors encoding RelA (10 μg), Tax (10 μg), and the indicated IκBβ construct (5 μg). After 48 h of growth, cells were arrested with CHX for 2 h. Nuclear extracts were prepared and analyzed in gel retardation assays. Arrows indicate the positions of nucleoprotein complexes containing RelA homodimers (upper arrow) and RelA/p50 heterodimers (lower arrow).

taining point mutations at all other potential phosphorylation sites in the N-terminal region of IκBβ, including alanine substitutions at Thr-10, Ser-45, and Thr-55 (mutants T10A, S45A, and T55A), or phenylalanine for Tyr-53 (mutant Y53F), failed to inhibit the Tax response.

To extend these findings, biochemical experiments were performed to monitor the stability of the S19/23A mutant of IκBβ when coexpressed with Tax in vivo. As shown in Fig. 3B, wild-type IκBβ (lane 2) and S19/23A (lane 3) were expressed at comparable levels in the cytoplasm of Tax-deficient cells. Furthermore, the S19/23A mutant retained the capacity to form stable cytoplasmic complexes with endogenous RelA (lane 6). Consistent with results shown in Fig. 2, steady-state levels of wild-type IκBβ were markedly reduced in Jurkat T cells expressing Tax (Fig. 3C, lane 2). However, the S19/23A mutant escaped from Tax-induced breakdown (lane 4). Taken together, these in vivo results confirmed that serines 19 and 23 of IκBβ are required for proteolytic inactivation by Tax.

We next performed gel retardation studies to determine whether the relative stabilities of these inhibitors in the presence of Tax correlated with the induction of nuclear NF-κB expression. Jurkat T cells were transfected with expression vectors encoding RelA, Tax, and either wild-type IκBβ or mutant S19/23A. Nuclear extracts were prepared from recipient cells and analyzed for κB-specific DNA binding activity in mobility shift assays. As shown in Fig. 3D (lanes 1 and 2), significant amounts of NF-κB were detected in nuclear extracts from control cells overexpressing RelA but lacking ectopic IκBβ. However, coexpression with either wild-type IκBβ (lane 3) or S19/23A (lane 5) blocked the formation of these nucleoprotein complexes. In cells expressing HTLV-1 Tax, the wild-type inhibitor was efficiently inactivated, as evidenced by the accumulation of nuclear NF-κB (lane 4). In contrast, Tax failed to mobilize NF-κB complexes containing the S19/23A mutant (lane 6). We conclude that Tax targets both IκBα and IκBβ for degradation through a related proteasome-dependent mechanism, which requires the presence of conserved serine residues in their N-terminal regions (13, 14, 78).

Differential expression of IκB proteins in HTLV-1-infected T lymphocytes. To assess the physiologic relevance of our observations with transfected cells, we next examined the functional interplay between IκBβ and Tax in HTLV-1-infected T lymphocytes. For these studies, we used, in addition to Jurkat T cells, five independent HTLV-1-transformed cell lines, SLB-1 (41), Hut102 (25), C8166 (60), MT-2 (48), and MT-4 (47). The SLB-1, Hut102, and MT-2 cell lines are chronically infected with HTLV-1 and produce infectious virions capable of transforming primary human T lymphocytes. The C8166 and MT-4 cells served as controls for Tax-specific effects. Whereas C8166 cells are infected with a defective HTLV-1 that selectively expresses Tax, MT-4 cells harbor a partially deleted HTLV-1 genome that fails to direct the synthesis of a functional Tax protein. In agreement with prior studies (42, 72), immunoblotting with peptide-specific antisera revealed the presence of two prominent isoforms of IκBα in cytoplasmic extracts from SLB-1, Hut102, C8166, and MT-2 cells (Fig. 4A, lanes 2 to 5) but not in MT-4 (lane 6) or uninfected Jurkat T (lane 1) cells. As shown in Fig. 4D, the more slowly migrating species was sensitive to treatment with calf intestinal phosphatase (lane 2) and thus likely represents the hyperphosphorylated form of IκBα that is modified at Ser-32 and Ser-36 (13, 14, 42, 72, 78). Consistent with previously published reports (13, 37, 42, 72), the presence of the hyperphosphorylated isoform of IκBα was dependent on Tax expression (Fig. 4C). In contrast to IκBα, the steady-state level of IκBβ protein was drastically reduced in each of the cell lines expressing Tax (Fig. 4B, lanes 2 to 5). However, IκBβ was readily detected in Tax-deficient cells (lanes 1 and 6). These results suggest that IκBβ protein expression is downregulated in HTLV-1-infected cells by a Tax-dependent mechanism.

The peptide aldehyde MG132 blocks NF-κB induction by preventing proteasome-mediated degradation of hyperphosphorylated IκBα (16, 53). To determine its effects on Tax-mediated turnover of endogenous IκBα and IκBβ, we treated HTLV-1-transformed cell lines with MG132 and monitored IκB expression by immunoblot analysis. As shown in Fig. 5A, incubation of C8166 cells with MG132 led to the rapid accumulation of the hyperphosphorylated form of IκBα (top panel). The observed increase in cytoplasmic IκBα protein was due to repression of Tax-mediated proteolysis rather than basal turnover, because Tax-deficient MT-4 cells maintained a constant steady-state level of IκBα over the same time course (Fig. 5A, bottom panel). In contrast, at doses sufficient to prevent proteolytic breakdown of ectopic IκBβ (Fig. 2C),

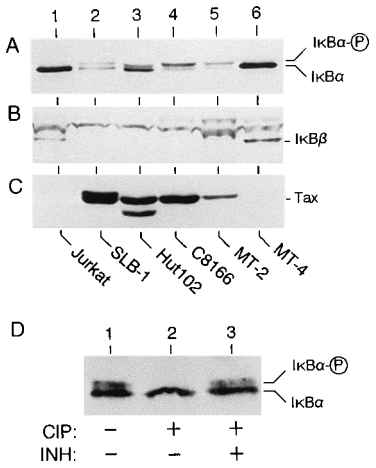

FIG. 4. IκBβ protein expression is downregulated in HTLV-1-infected T lymphocytes. Equivalent amounts of cytoplasmic protein (100 μg) from either uninfected Jurkat T cells (lane 1) or the indicated HTLV-1-infected T-cell lines (lanes 2 to 6) were resolved by SDS-PAGE and analyzed by immunoblotting with a peptide-specific antibody raised against either IκBα (amino acids 289 to 317; A), IκBβ (amino acids 330-359; B), or Tax (C). Similar results were obtained when an independent IκBβ-specific antiserum generated against the full-length inhibitor was used. (D) Cytoplasmic extracts from MT-2 cells were incubated in the presence (lanes 2 and 3) or absence (lane 1) of calf intestinal phosphatase (CIP) and then subjected to immunoblot analysis with an IκBα-specific antiserum. Control reactions (lane 3) were supplemented with a previously described cocktail of phosphatase inhibitors (INH) (13).

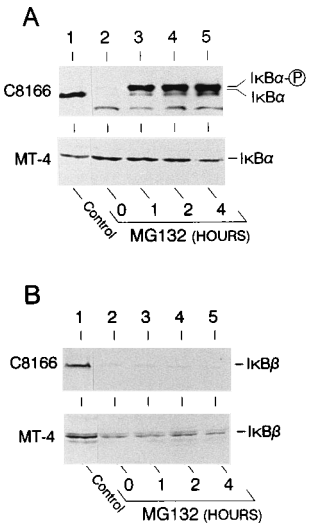

FIG. 5. Inhibition of proteasome activity in HTLV-1-infected lymphocytes leads to the accumulation of IκBα but not IκBβ. (A) The HTLV-1-infected T-cell lines C8166 (upper panel) and MT-4 (lower panel) were treated with the proteasome inhibitor MG132 (100 μM) for the indicated periods of time. Cytoplasmic extracts were prepared and analyzed by immunoblotting with peptide-specific IκBα antisera (lanes 2 to 5). Equivalent amounts of cytoplasmic protein from Jurkat T cells were analyzed in parallel (lane 1) as a control for basal levels of IκBα expression in uninfected T lymphocytes. Major immunoreactive IκB species are indicated. The contrasting steady-state expression of IκBα in untreated C8166 cells (lane 2) relative to that shown in Fig. 4 (lane 4) reflects differences in total protein input and film exposure times. (B) C8166 (upper panel) and MT-4 (lower panel) cells were treated with MG132 for the indicated periods of time. Cytoplasmic proteins were processed as described above and immunoblotted with IκBβ-specific antiserum generated against the full-length inhibitor (lanes 2 to 5). Control experiments performed with equivalent amounts of Jurkat T cell extracts are shown in lane 1.

MG132 failed to affect the cytoplasmic pool of endogenous IκBβ (Fig. 5B). These results with endogenous IκBβ were recapitulated in assays using three independent Tax-expressing cell lines, SLB-1, Hut102, and MT-2 (data not shown). The capacity of MG132 to mediate the rapid accumulation of endogenous IκBα but not IκBβ suggests that these two inhibitors are synthesized at different rates in the presence of Tax.

HTLV-1 Tax transcriptionally induces IκBα but not IκBβ. During normal T-cell activation, a transient NF-κB response is maintained by an autoregulatory mechanism that involves NF-κB-directed transcription of the IκBα gene (8, 15, 17, 20, 30, 36, 43, 73). Although the *cis*-acting elements that regulate the IκBβ gene remain undefined, a recent report suggests that this transcription unit is not under NF-κB control (77). On the basis of these prior observations, we reasoned that the distinct patterns of IκB expression observed in HTLV-1-infected cells (Fig. 4 and 5) may reflect the capacity of Tax to selectively stimulate NF-κB-dependent transcription of the IκBα gene. To test this hypothesis, we examined the steady-state levels of IκBα and IκBβ transcripts in HTLV-1-transformed cell lines. As shown in Fig. 6A, IκBα mRNA pools were dramatically elevated in virally infected cells expressing Tax (lanes 2 to 4) relative to the level observed in Tax-deficient MT-4 cells (lane 1). In sharp contrast to IκBα, comparable amounts of IκBβ mRNA were detected in each of these transformed cell lines (Fig. 6A, middle panel).

Experiments were next performed to determine whether the NF-κB-activating function of Tax is required to elicit these transcriptional responses. For these studies, we examined the steady-state levels of IκB transcripts in Jurkat T cells following transfection with plasmids encoding wild-type Tax (Tax-WT) or mutants of Tax that are unable to stimulate either the CREB/ATF (Tax-M47) or the NF-κB/Rel (Tax-M22) transcription factor pathway (68). As shown in Fig. 6B, IκBα but not IκBβ mRNA expression was markedly upregulated in cells expressing either Tax-WT (lane 2) or Tax-M47 (lane 4) relative to control transfectants (lane 1). These stimulatory effects were dependent on the NF-κB-inducing function of Tax, since Tax-M22 failed to enhance IκBα mRNA expression (lane 3). Consistent with these findings, the level of IκBα transcripts was significantly increased in cells expressing the RelA transactivating subunit of NF-κB (Fig. 6C, lane 2) but not the p50

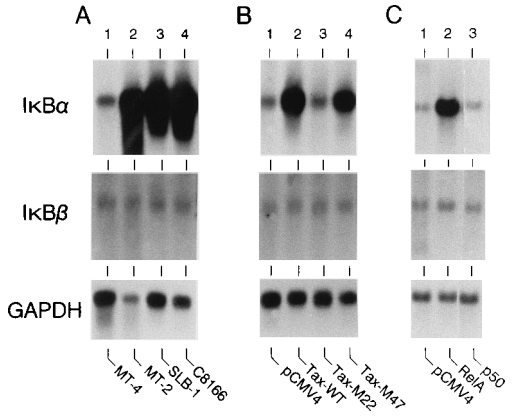

FIG. 6. Northern blot analysis of IκB mRNA transcripts. Poly(A)$^+$ RNA was prepared from HTLV-1-infected T cell lines (A), human Jurkat T cells transfected with the indicated Tax effector plasmids (B), or transfectants overexpressing the indicated NF-κB subunits (C). In all cases, poly(A)$^+$ RNA was selected from ~750 μg of total RNA. Following fractionation on formaldehyde-agarose gels, RNA was transferred to Zeta Probe membranes and sequentially hybridized with a ^{32}P-labeled cDNA probe (~10^6 cpm/ml) for IκBα, IκBβ, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

subunit (lane 3), which lacks a transcriptional activation domain (6, 58, 63). These results confirmed that the NF-κB-inducing activity of Tax accounts for the high-level expression of IκBα mRNA in HTLV-1-infected lymphocytes. Furthermore, these findings establish a strong correlation between the chronic downregulation of IκBβ protein expression and the failure of Tax to induce IκBβ gene transcription in virus-infected cells.

DISCUSSION

The Tax protein of HTLV-1 is a potent transcriptional activator with demonstrated oncogenic potential (31, 50, 55, 69, 70, 75). This transforming protein has the capacity to activate the functional expression of various members of the NF-κB/Rel family of transcription factors (5, 44, 59). Recent studies with mice transgenic for the *tax* gene suggest that the constitutive pattern of NF-κB induced in HTLV-1-infected cells is required to maintain the transformed phenotype (40). We have focused on defining how Tax subverts the action of NF-κB in $CD4^+$ T lymphocytes, the in vivo target for HTLV-1-induced transformation. Insights into the precise nature of this virus-host interaction have been complicated by the capacity of Tax to assemble with multiple components of the NF-κB pathway (10, 32, 33, 81). However, recent experiments have revealed a direct mechanistic link between the abilities of Tax to activate NF-κB and to stimulate degradation of IκBα by the 26S proteasome (13, 62). We demonstrate here that Tax also induces proteasome-dependent degradation of IκBβ, a recently cloned member of the IκB family of NF-κB inhibitors (77). Thus, Tax mobilizes not one but two distinct pools of cytoplasmic NF-κB/Rel complexes to the nucleus. The dual specificity of Tax for IκBα and IκBβ likely contributes to the mechanism by which HTLV-1 deregulates the expression of growth-related genes under NF-κB control (5, 19, 27, 39, 44, 59).

During a normal immune response, IκBα is rapidly phosphorylated and then completely degraded via the ubiquitin-proteasome pathway (13, 14, 16, 62, 78, 79). Recent studies have suggested that signal-dependent phosphorylation of IκBα at serines 32 and 36 is a prerequisite for proteolytic breakdown (16). Two lines of experimental evidence indicate that a related posttranslational process leads to Tax-mediated inactivation of IκBα and IκBβ by the proteasome. First, treatment of T-cell transfectants with MG132, a peptide aldehyde inhibitor of the proteasome, blocks Tax-induced breakdown of both IκBα (13) and IκBβ (Fig. 2C). Degradation of IκBβ in Tax-expressing cells was also prevented by the proteasome inhibitor lactacystin (Fig. 2D), which exhibits a more restricted range of action and has no detectable effect on calpain or cathepsin B (21). Second, by analogy with IκBα (13), disruption of two conserved serine residues positioned within the amino terminus of IκBβ yields a dominant-negative inhibitor of NF-κB that is resistant to proteolytic breakdown in the presence of Tax (Fig. 3). Taken together, these findings suggest that HTLV-1 Tax mimics extracellular immune signals which target IκB proteins to the proteasome, including those mediated by cytokine and antigen receptors (13, 14, 16, 62, 78, 79).

The mechanism by which Tax accesses these host signaling pathways for IκB inactivation remains elusive. Given the recent finding that Tax forms stable complexes with IκBα when overexpressed in vivo (33, 74), it is possible that this viral protein initiates the process of NF-κB induction by physically dissociating IκB proteins from latent cytoplasmic complexes. Indeed, since free IκBα is rapidly degraded (73), this model could potentially explain the mechanism by which Tax stimulates IκB turnover. However, we have observed that the Tax-M22 mutant, which is defective for NF-κB induction (68) and IκBα breakdown (13), retains the capacity to bind IκBα in transfected cells. In addition wild-type Tax is fully competent to form stable complexes with dominant-negative forms of IκBα that escape from Tax-dependent proteolysis in vivo (data not shown). Assuming the interaction is physiologically relevant, these findings suggest that Tax binding to IκBα is not sufficient to target the inhibitor to the proteasome. An alternative view is that Tax regulates the turnover of IκB proteins by influencing the phosphorylation status of serine residues positioned within their N-terminal regions. In light of emerging evidence that Tax and immune activation signals converge on the same phosphoryl group acceptors within IκBα (13, 14, 78), this coupling mechanism may involve the action of a common protein kinase(s). Consistent with this proposal, antioxidants that prevent signal-dependent phosphorylation of IκBα in response to normal physiologic cues also inhibit Tax-mediated induction of NF-κB (64, 65, 79). However, we cannot exclude the possibility that this modification step is dependent on the formation of IκB-Tax complexes.

Despite the destabilizing effects of Tax on both IκBα and IκBβ at the protein level, this viral pathway for NF-κB activation diverges downstream of IκB degradation at the transcription level, resulting in NF-κB-directed synthesis of IκBα but not IκBβ (Fig. 6). In HTLV-1-infected T lymphocytes, the capacity of Tax to inactivate IκBβ without stimulating transcription of the corresponding gene culminates in the chronic downregulation of IκBβ protein expression. In contrast, the cytoplasmic pool of IκBα is maintained by de novo protein synthesis in response to NF-κB activation (Fig. 4 and 5). This pattern of IκB expression, coupled with the tonic inducing action of HTLV-1 Tax, may account for the persistent NF-κB response in virally infected cells. In this regard, Thompson et al. recently reported that IκBβ inactivation is tightly associated with nonviral agents that induce a persistent NF-κB response, such as LPS and IL-1 (77). In cells treated with either of these extracellular inducers, both IκBα and IκBβ are rapidly degraded, but only IκBα reaccumulates in the cytoplasm following NF-κB induction. In contrast, IκBα appears to be selectively degraded in cells treated with tumor necrosis factor alpha or phorbol esters, both of which elicit a transient NF-κB response (77). These findings collectively suggest that IκBβ plays a significant role in regulating the duration of NF-κB activity in the nuclear compartment.

Why might inactivation of IκBβ be required for the development of a chronic NF-κB response? One possibility is that IκBβ-bound NF-κB is uniquely endowed for persistent nuclear expression. For example, following its release from IκBβ, NF-κB may be refractory to postinduction repression mediated by newly synthesized IκBα (22, 77). Alternatively, the findings presented here may reflect the requirement for a threshold level of cytoplasmic IκB to fully inhibit NF-κB. While IκB expression is downregulated in HTLV-1-infected lymphocytes, the concentration of certain Rel subunits is elevated as a result of NF-κB-directed transcription of the corresponding genes (37, 72, 76). As a consequence of this stoichiometric imbalance, the total intracellular concentration of IκB may be insufficient to sequester NF-κB in the cytoplasmic compartment of Tax-expressing cells.

The proteasome is rapidly emerging as a key regulator of transcription factors involved in the control of normal cellular growth. In addition to IκB proteins (22), proteasome-dependent turnover of the c-*jun* proto-oncogene product appears to play a pivotal role in governing its transcriptional activity (80). Furthermore, recent studies suggest that human papillomavirus types 16 and 18 promote cellular transformation by stim-

ulating proteasome-mediated breakdown of the p53 tumor suppressor (61). It is now evident that HTLV-1 also employs the proteasome to constitutively activate NF-κB in $CD4^+$ T lymphocytes, resulting in the abnormal expression of select genes involved in growth-signal transduction (27). Our prior findings (13, 62) and the results presented here suggest that this mechanism involves the unique capacity of the HTLV-1 Tax protein to convert both IκBα and IκBβ into labile proteasome substrates. Future studies to define precisely how Tax executes this IκB targeting function may illuminate novel strategies for therapeutic control of HTLV-1-associated diseases.

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