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Human T Cell Leukemia Virus Type I Tax–Induced IκB-ζ Modulates Tax-Dependent and Tax-Independent Gene Expression in T Cells[1]

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Abstract

Human T cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T cell leukemia (ATL) and various inflammatory disorders including HTLV-I–associated myelopathy/tropical spastic paraparesis. HTLV-I oncoprotein Tax is known to cause permanent activation of many cellular transcription factors including nuclear factor-κB (NF-κB), cyclic adenosine 3′,5′-monophosphate response element–binding protein, and activator protein 1 (AP-1). Here, we show that NF-κB–binding cofactor inhibitor of NF-κB-ζ (IκB-ζ) is constitutively expressed in HTLV-I–infected T cell lines and ATL cells, and Tax transactivates the *IκB-ζ* gene, mainly through NF-κB. Microarray analysis of IκB-ζ–expressing uninfected T cells demonstrated that IκB-ζ induced the expression of NF-κB– and interferon-regulatory genes such as *B cell CLL/lymphoma 3* (*Bcl3*), *guanylate-binding protein 1*, and *signal transducer and activator of transcription 1*. The transcriptional activation domain, nuclear localization signal, and NF-κB–binding domain of IκB-ζ were required for Bcl3 induction, and IκB-ζ synergistically enhanced Tax-induced Bcl3 transactivation in an NF-κB–dependent manner. Interestingly, IκB-ζ inhibited Tax-induced NF-κB, AP-1 activation, and HTLV-I transcription. Furthermore, IκB-ζ interacted with Tax *in vitro* and this interaction was also observed in an HTLV-I–transformed T cell line. These results suggest that IκB-ζ modulates Tax-dependent and Tax-independent gene transcription in T cells. The function of IκB-ζ may be of significance in ATL genesis and pathogenesis of HTLV-I–associated diseases.

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Abbreviations: Abs, antibodies; AP-1, activator protein 1; ATL, adult T cell leukemia; Bcl3, B cell CLL/lymphoma 3; CBB, Coomassie brilliant blue; CREB, cyclic adenosine 3′,5′-monophosphate response element–binding protein; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBP, guanylate-binding protein; GST, glutathione *S*-transferase; HTLV-I, human T cell leukemia virus type I; IFN, interferon; IκB-ζ, inhibitor of NF-κB-ζ; IKK, IκB kinase; IL-2Rα, interleukin-2 receptor α chain; iNOS, inducible nitric oxide synthase; ISGs, IFN-stimulated genes; ISRE, IFN-α–stimulated response element; LLnL, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; LTR, long terminal repeat; NF-κB, nuclear factor-κB; NIK, NF-κB–inducing kinase; NEs, nuclear extracts; NLS, nuclear localization signal; PBMCs, peripheral blood mononuclear cells; Rb, retinoblastoma protein; RT-PCR, reverse transcription–polymerase chain reaction; shRNA, short hairpin RNA; SLAMF7, SLAM family member 7; STAT1, signal transducer and activator of transcription 1; TAD, transcriptional activation domain; WCEs, whole-cell extracts; WT, wild-type

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Introduction

Adult T cell leukemia (ATL) is a highly aggressive malignancy of $CD4^+$ T cells caused by human T cell leukemia virus type I (HTLV-I) [1]. Infection with this retrovirus also results in inflammatory disorders including HTLV-I–associated myelopathy/tropical spastic paraparesis [2]. The majority of infected persons remain clinically asymptomatic, whereas only 2% to 5% develop neoplasia after a latency of 40 to 60 years, which develops through genetic and epigenetic changes in the cell [3]. However, the exact pathogenic mechanisms involved in leukemogenesis remain obscure.

Tax, the viral oncoprotein, plays a central role in tumorigenesis and contributes to the pathogenesis of ATL by inducing activation of many cellular transcription factors including nuclear factor-κB (NF-κB), cyclic adenosine 3′,5′-monophosphate response element–binding protein (CREB), and activator protein 1 (AP-1) [4]. Notably, NF-κB activation is essential for cellular transformation by HTLV-I [5]. Although Tax is critical in the early stages of leukemogenesis, it also elicits a strong cytotoxic T lymphocyte response resulting in rapid targeting of Tax-expressing cells for their elimination. To escape from cytotoxic T lymphocytes, Tax is not expressed in ATL cells, probably due to the deletion or DNA methylation of a 5′ long terminal repeat (LTR) and genetic changes in the *tax* gene, which inactivate its functions [3]. However, NF-κB is constitutively activated in primary ATL cells [6]. These facts suggest activation of NF-κB in HTLV-I–infected T cells and ATL cells in Tax-dependent and Tax-independent manners.

One of the inhibitor of NF-κB (IκB) family proteins, IκB-ζ, is an inducible nuclear protein [7–9]. IκB-ζ is induced by proinflammatory stimuli and lipopolysaccharide in NF-κB– and CREB-dependent manners [10,11]. During inflammatory responses, IκB-ζ positively or negatively regulates NF-κB–mediated transcription [12,13]. While the inducible expression mechanisms and functions of IκB-ζ in the immune system have been thoroughly investigated, the role of constitutive expression of IκB-ζ in tumorigenesis and pathogenesis of various cancers, including ATL, remains elusive. In this study, we investigated IκB-ζ expression in HTLV-I–infected T cells and ATL cells and the mechanism for *IκB-ζ* gene transactivation by Tax. In addition, we studied the roles played by inducible IκB-ζ as a means for regulating Tax-dependent and Tax-independent cellular gene expression.

Materials and Methods

Cells

All the human T cell lines described previously [14–22] were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers and patients with ATL using Ficoll-Paque density gradient centrifugation (GE Healthcare, Piscataway, NJ). Informed consent was obtained from all blood and tissue donors.

Antibodies and Reagents

Antibodies (Abs) to IκB-ζ for Western blot and immunohistochemical analyses were purchased from Cell Signaling Technology (Beverly, MA) and Novus Biologicals (Littleton, CO), respectively. The following Abs were used for Western blot analysis: anti–B cell CLL/lymphoma 3 (Bcl3; Bio Matrix Research Inc, Nagareyama, Japan), anti–guanylate-binding protein 1 (GBP-1) to anti–GBP-5 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin, anti–retinoblastoma protein (Rb; NeoMarkers, Fremont, CA), and anti-FLAG (Sigma-Aldrich, St Louis, MO). Mouse monoclonal Ab to Tax, Lt-4 [23], was used for Western blot analysis and immunoprecipitation. Abs to p50, RelA, c-Rel, p52, and RelB for electrophoretic mobility shift assay (EMSA) were purchased from Santa Cruz Biotechnology. Bay 11-7082 and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) were purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich, respectively.

Reverse Transcription–Polymerase Chain Reaction

Reverse transcription–polymerase chain reaction (RT-PCR) was carried out as described previously [24]. The sequences of the primers for IκB-ζ, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [25], interleukin-2 receptor α chain (IL-2Rα) [26], Bcl3 [27], GBP-1 [28], signal transducer and activator of transcription 1 (STAT1) [29], SLAM family member 7 (SLAMF7) [30], Tax in HTLV-I–infected T cell lines and ATL cells [25], and Tax in JPX-9 cells [25] are summarized in Table 1. PCR was halted during the exponential phase of DNA amplification, and the reaction products were fractionated on agarose gels and visualized by ethidium bromide staining. The obtained bands of amplified DNA were quantified using ImageJ.

Plasmids

pGL3-basic and phRL-TK were purchased from Promega (Madison, WI). pGL3–hIκB-ζ (–11 kb) and pGL3–hIκB-ζ (–853) were described previously [10]. pGL3–hIκB-ζ (–853) κB1D, κB2D, and κB1/κB2D were prepared from pGL3–hIκB-ζ (–853). Bcl3, GBP-1, and STAT1 luciferase reporter constructs were described previously [31–33]. IL-8 –133-Luc [34], pGL3–inducible nitric oxide synthase (iNOS) [35], NF-κB–Luc [36], AP-1–Luc [34], and HTLV-I LTR-Luc [37] were used for reporter assay. Expression vectors for Tax and its mutants and the dominant negative mutants of IκBα, IκBβ, IκB kinase 1 (IKK1; IKKα), IKK2 (IKKβ), NEMO (IKKγ), and NF-κB–inducing kinase (NIK) were described previously [38–42]. The dominant

Table 1. Primer Sequences.

Gene Name	Forward Sequence	Reverse Sequence
Human IκB-ζ	5′-GGAGCTTTTACTGAAGAATAAGA-3′	5′-ATCTGTTCTCCCACAGGGCCATC-3′
Human GAPDH	5′-GCCAAGGTCATCCATGACAACTTTGG-3′	5′-GCCTGCTTCACCACCTTCTTGATGTC-3′
Human IL-2Rα	5′-ATCCCACACGCCACATTCAAAGC-3′	5′-TGCCCCACCACGAAATGATAAAT-3′
Human Bcl3	5′-CCACAGACGGTAATGTGGTG-3′	5′-TATTGCTGTGGTGCAGGGTA-3′
Human GBP-1	5′-GGTCCAGTTGCTGAAAGAGC-3′	5′-TGACAGGAAGGCTCTGGTCT-3′
Human STAT1	5′-AAGGTGGCAGGATGTCTCAGT-3′	5′-TGGTCTCGTGTTCTTCTGTTCTG-3′
Human SLAMF7	5′-GTCTCTTTGTACTGGGGCTATTTC-3′	5′-TTTTCCATCTTTTTCGGTATTT-3′
Tax in HTLV-I–infected T cell lines and primary ATL cells	5′-CCGGCGCTGCTCTCATCCCGGT-3′	5′-GGCCGAACATAGTCCCCCAGAG-3′
Tax in JPX-9 cells	5′-ATCGGCTCAGCTCTACAGTTCCT-3′	5′-ATTCGCTTGTAGGGAACATTGGT-3′

negative mutants of CREB and pcDNA3 were purchased from Clontech Laboratories (Mountain View, CA) and Invitrogen (Carlsbad, CA), respectively.

pcDNA3-HBZ-Myc, pcDNA3-RelA, and various mouse IκB-ζ–expressing vectors were described previously [13,43,44]. pCS-puro-EGFP, pCS-puro-Zeta#1, pCS-puro-Zeta#2, pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev were used for IκB-ζ knockdown by lentiviral induction. Oligonucleotides containing the targeting pSR-enhanced green fluorescent protein (EGFP) (5′-GCAGCACGACTTCTTCAAG-3′) and human IκB-ζ No. 1 (5′-GACACCACCTCAAACACCA-3′) and No. 2 (5′-GACCAGGCTTCCCTGTACC-3′) were inserted into the pSUPER.retro.puro vector (Oligoengine, Seattle, WA), generating pSR-enhanced green fluorescent protein (EGFP), pSR-Zeta#1, and pSR-Zeta#2, respectively. The short hairpin RNA (shRNA) expression cassettes were then transferred to a lentiviral vector, pCS-puro-PRE [45]. Lentiviral vectors expressing shRNA were constructed by inserting a fragment of pSR-EGFP, Zeta#1, and Zeta#2 into pCS-puro-PRE, denoted as pCS-puro-EGFP, Zeta#1, and Zeta#2, respectively. The *Escherichia coli* expression vectors pGEX 4T-2 and pGEX 4T-2-Tax [46] were used for the purification of glutathione *S*-transferase (GST) and GST-Tax, respectively.

Electrophoretic Mobility Shift Assay

Nuclear extracts (NEs) from cells were prepared, and EMSA was performed as described previously [24]. The DNA sequences of probes and competitors are summarized in Table 2.

Immunohistochemical Analysis

Biopsy samples were taken from the lymph nodes of six patients with ATL and also from two specimens of normal lymph nodes. Immunohistochemical staining was performed using anti–IκB-ζ after pretreatment of the deparaffinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA). The sections were counterstained with methyl green, and the stained cells were examined under a light microscope.

Luciferase Assay

Cells were transfected with the appropriate reporter and effector plasmids by electroporation using Gene Pulser (Bio-Rad, Hercules, CA). After 24 hours, luciferase assays were performed with the Dual Luciferase Assay System (Promega). Luciferase activities were normalized relative to the *Renilla* luciferase activity from cotransfected phRL-TK.

Western Blot Analysis and Coomassie Brilliant Blue Staining

Western blot analysis was performed using a standard protocol [24]. As loading control, expression of actin and Rbs was included. The bands were prepared with Coomassie brilliant blue (CBB) staining.

Table 2. DNA Sequences of Probes and Competitors.

Name of Oligonucleotide	Top Strand Sequence
IκB-ζ WT κB1	5′-GATCCGACGGGAATGTCCGGGACT-3′
IκB-ζ WT κB2	5′-GATCGGTCTGGGAATTTCCAGTG-3′
IκB-ζ κB1 mutant	5′-GATCCGACGtGtATGaCCGGGACT-3′
IκB-ζ κB2 mutant	5′-GATCGGTCTGtGtATaaCCAGTG-3′
IL-2Rα κB	5′-GATCCGGCAGGGGAATCTCCCTCTC-3′
IL-8 AP-1	5′-GATCGTGATGACTCAGGTT-3′
Oct-1	5′-GATCTGTCGAATGCAAATCACTAGAA-3′

The underlined sequences are the NF-κB–, AP-1–, and Oct-1–binding sites, respectively. The sites of mutation are indicated in lowercase.

Microarray Analysis

RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Microarray analysis using a Human 1A Oligo Microarray Kit V2 (Agilent Technologies, Santa Clara, CA) was performed as described previously [24].

IκB-ζ Knockdown

The procedure of gene knockdown by lentiviral induction was described previously [45]. Viral supernatants were generated by transfecting 293T cells with pCS-puro-EGFP, pCS-puro-Zeta#1, or pCS-puro-Zeta#2 together with the pCAG-HIVgp and pCMV-VSV-G-RSV-Rev using FuGENE HD (Promega).

Immunoprecipitation and GST Pull-Down Assay

293T cells were cotransfected with various expression plasmids for IκB-ζ and/or pHβ-Tax using FuGENE HD. In the immunoprecipitation assay, anti-Tax Ab and Protein G Sepharose 4 Fast Flow (GE Healthcare) were used. For GST pull-down assay, 293T cells were lysed in lysis buffer, and whole-cell extracts (WCEs) were incubated with glutathione sepharose 4B (GE Healthcare)–conjugated GST or GST-Tax proteins purified from isopropyl thiogalactoside–induced *E coli* JM109 transformed with pGEX 4T-2 or pGEX 4T-2–Tax [46].

Statistical Analysis

Differences between groups were examined for statistical significance using the unpaired Student's *t* test. *P* values less than .05 denoted the presence of a statistically significant difference.

Results

Constitutive Expression of IκB-ζ in HTLV-I–Infected T Cells and ATL Cells

RT-PCR identified marked expression of IκB-ζ in Tax-expressing HTLV-I–transformed T cell lines, MT-2, MT-4, C5/MJ, SLB-1, and HUT-102, compared with uninfected T cell lines, Jurkat, Molt-4, and CCRF-CEM (Figure 1*A*). IκB-ζ was also weakly expressed in ATL-derived T cell lines, MT-1, TL-OmI, and ED-40515(−). IκB-ζ, a known nuclear protein (Figure 1*B*), was also highly expressed in the nuclei of HTLV-I–transformed T cell lines, MT-2, SLB-1, HUT-102, and MT-4. RT-PCR showed high expression of IκB-ζ in PBMCs from patients with ATL compared with those from healthy donors (Figure 1*C*). In addition, immunohistochemical staining identified IκB-ζ–positive ATL cells in the nuclei of lymph nodes obtained from all six patients with ATL (Figure 1*D*). These data suggest that IκB-ζ is constitutively expressed in HTLV-I–infected T cells and ATL cells.

Tax Transactivates the IκB-ζ Promoter Mainly through the NF-κB Pathway

To study the effect of Tax on *IκB-ζ* gene expression at the transcriptional level, we performed luciferase assays in Jurkat cells using reporter plasmids for IκB-ζ. Tax markedly activated both reporter plasmids, IκB-ζ (−11 kb) and IκB-ζ (−853), in a dose-dependent manner (Figure 2*A*). HBZ, another viral regulatory protein, showed no effect on the activation of the IκB-ζ promoter in contrast to Tax (Figure 2*B*). To narrow down the transactivation-relevant signaling pathways, Tax M22 and 703 mutants [38] were cotransfected along with the IκB-ζ promoter construct, followed by determination of luciferase activities (Figure 2*C*). Tax M22 mutant, defective in NF-κB activation, failed

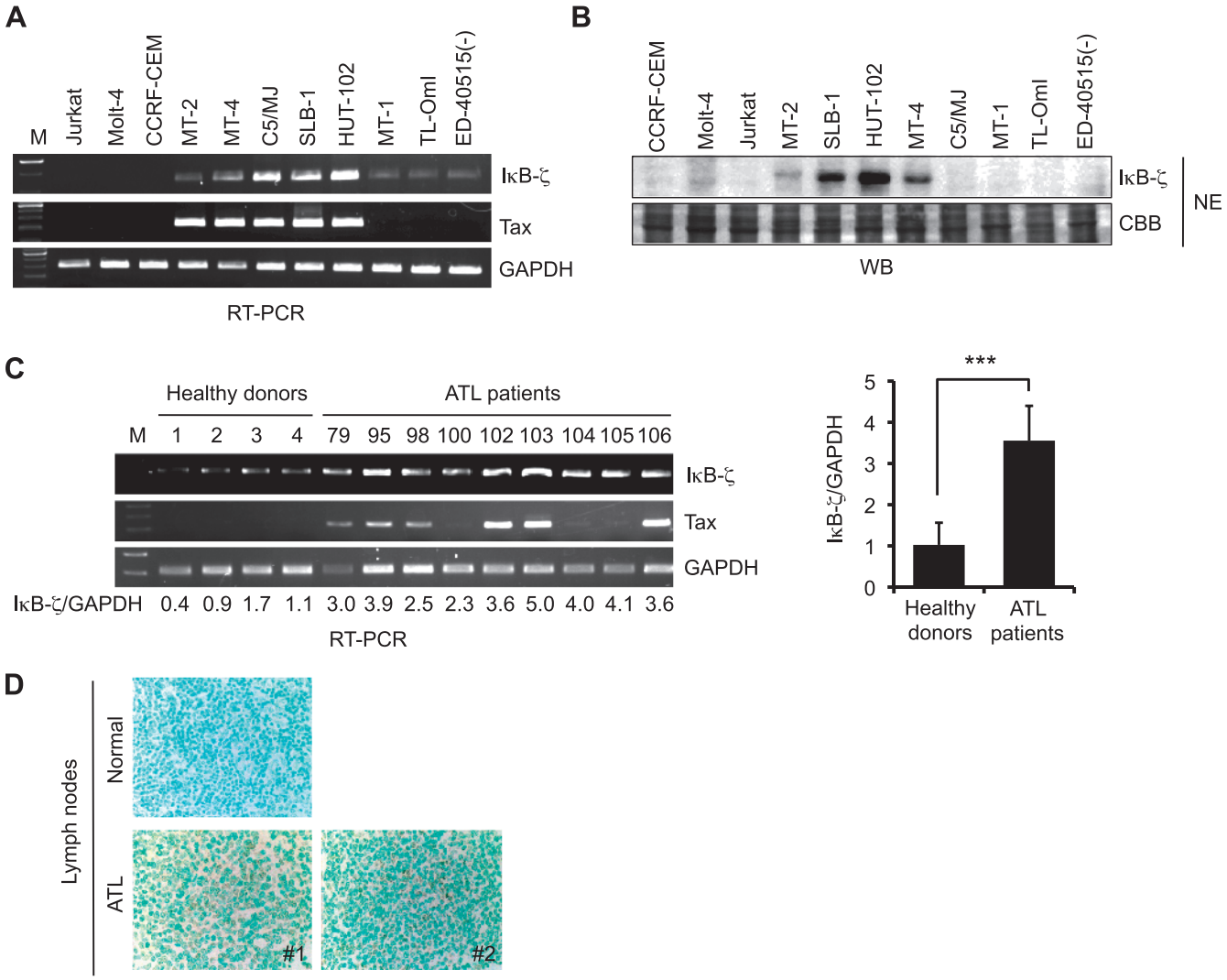

Figure 1. Constitutive expression of IκB-ζ in HTLV-I–infected T cell lines and primary ATL cells. (A) Expression of IκB-ζ and Tax mRNA in HTLV-I–infected T cell lines. RT-PCR analysis was carried out for IκB-ζ, Tax, and GAPDH (loading control). (B) Expression of IκB-ζ in NEs from HTLV-I–infected T cell lines. Western blot analysis for IκB-ζ was performed by immunoblot analysis with anti–IκB-ζ Ab. CBB staining was carried out for loading control. (C) RT-PCR analysis for IκB-ζ, Tax, and GAPDH expression in PBMCs from healthy donors and patients with ATL. IκB-ζ/GAPDH ratios were calculated by densitometric analysis of the bands. Data on the right are means ± SD of ratios (***$P < .001$). (D) Immunohistochemical staining for IκB-ζ in ATL lymph nodes. Tissue sections from ATL lymph nodes and normal lymph nodes were stained with anti–IκB-ζ Ab. Representative results from two patients with ATL and one normal lymph node. Original magnification, ×400.

to activate the IκB-ζ promoter. In contrast, Tax 703 mutant, which can activate NF-κB but not CREB, slightly activated the IκB-ζ promoter, but the level was less than wild-type (WT) Tax. However, Tax M22 and 703 mutants together activated the IκB-ζ promoter as observed with WT Tax expression. These results suggest that Tax activates the IκB-ζ promoter in NF-κB– and CREB-dependent manners. Next, we examined whether Tax-mediated transactivation of the *IκB-ζ* gene requires the signal transduction components involved in NF-κB activation. The dominant interfering mutants of IκBα, IκBβ, and NEMO and kinase-deficient mutants of IKK1, IKK2, and NIK were tested for their ability to inhibit Tax-mediated transactivation of IκB-ζ reporter gene activity. The expression of these mutants, especially IκBα, IκBβ, and IKK1 inhibitory mutants, significantly inhibited Tax-induced activation of the IκB-ζ promoter (Figure 2*D*), suggesting that signaling components involved in the activation of NF-κB are necessary for Tax-mediated transactivation of IκB-ζ promoter. In addition, we tested the potential role of CREB in modulating Tax-induced IκB-ζ promoter activity. For this purpose, the dominant negative CREB mutants, KCREB and CREB133, were transfected with IκB-ζ promoter construct as well as an expression vector for Tax. KCREB contains mutations in its DNA-binding domain [47]. CREB133 contains a serine-to-alanine mutation corresponding to amino acid 133, and this mutation blocks CREB phosphorylation, thus preventing transcription [47]. Overexpression of CREB133, but not KCREB, inhibited the IκB-ζ promoter activity in response to Tax by about 50% (Figure 2*E*), suggesting that CREB phosphorylation is partially required for Tax-mediated IκB-ζ promoter activation.

The human IκB-ζ promoter (position, −853 to −17) contains two NF-κB–binding sequences [10], κB1 and κB2 sites (Figure 2*F*). A single deletion of the κB2 site from the IκB-ζ reporter plasmid markedly inhibited either Tax- or RelA-induced transactivation, whereas a single deletion of the κB1 site resulted in moderate activation (Figure 2*F*).

Double deletions completely abolished the Tax- and RelA-induced transactivation. These results suggest that both NF-κB–binding sites in the promoter are essential for the complete transcriptional activation of the *IκB-ζ* gene.

NF-κB–Binding Activity in the Two NF-κB–Binding Sites of the IκB-ζ Promoter

In the next step, we identified the transcription factors that bind to the κB1 and κB2 elements in the IκB-ζ promoter. EMSA was performed with oligonucleotides representing the κB1 and κB2 elements. Protein complexes binding to the κB2 sites were detected using NEs derived from HTLV-I–transformed T cell lines and ATL-derived T cell lines compared with uninfected T cell lines, although the levels of binding activities with ATL-derived T cell lines were less than those with transformed T cell lines (Figure 3*A*). In addition, protein complexes binding to the κB1 sites were detected with NEs derived from transformed T cell lines.

The specificity of DNA-protein complex formation was assessed by competition studies with unlabeled competitors. As expected, "cold" (unlabeled) IκB-ζ WT κB2 and κB1 oligonucleotides or one based

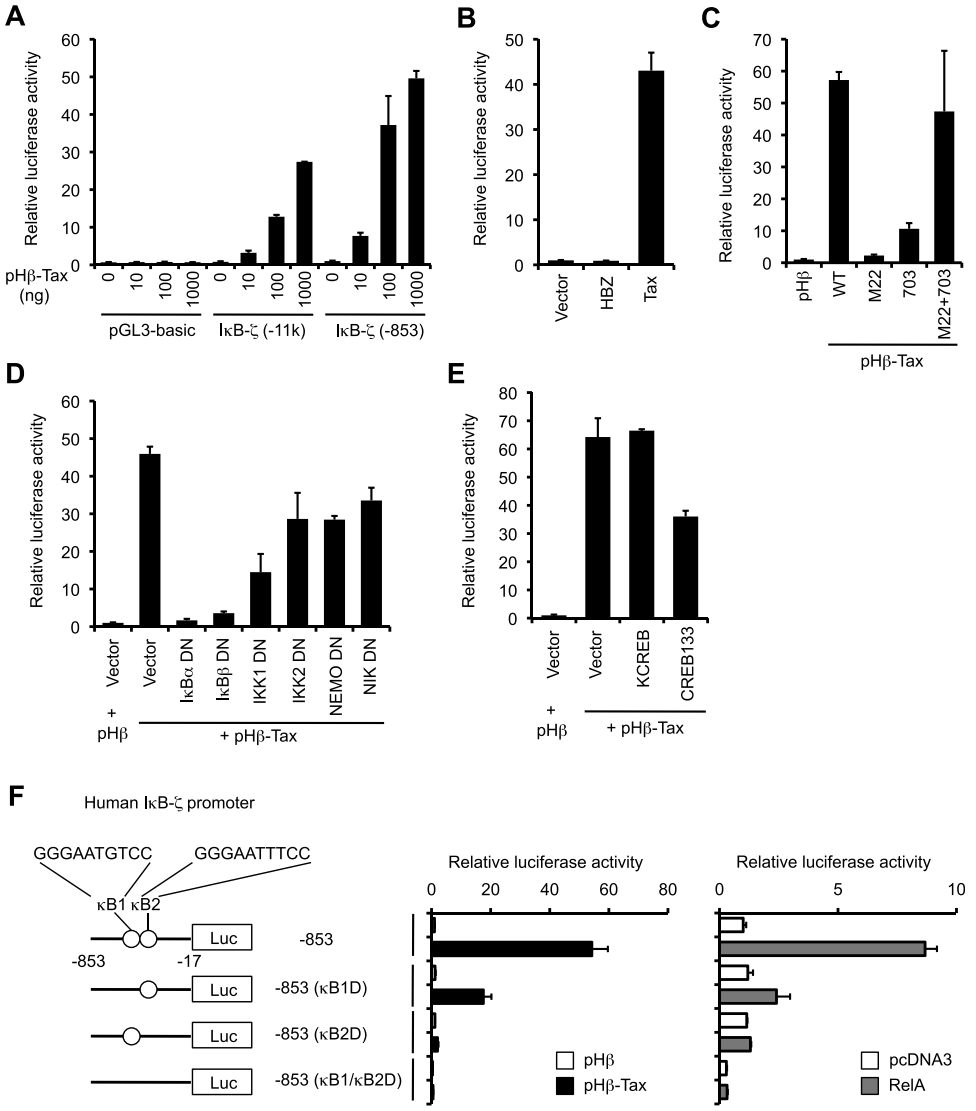

Figure 2. Tax transactivates the IκB-ζ promoter mainly through the NF-κB pathway. (A) Jurkat cells were transfected with the indicated IκB-ζ promoter fragments cloned into pGL3-basic together with various amounts of pHβ-Tax. Cells were harvested 24 hours post-transfection, and luciferase activity was measured. The activities are expressed relative to that of cells transfected with pGL3-basic alone, which was defined as 1. (B) Cells were transfected with pGL3–IκB-ζ (−853) together with empty vector, pcDNA3.1-HBZ-Myc, or pHβ-Tax. The results are expressed as fold induction by Tax or HBZ relative to the vector alone. (C) Cells were transfected with pGL3–IκB-ζ (−853) together with pHβ empty vector, pHβ–wild-type Tax (WT), Tax M22 mutant, Tax 703 mutant alone, or the combination of Tax M22 and Tax 703 mutants. The results are expressed as fold induction by Tax or Tax mutants relative to the vector alone. (D and E) Cells were transfected with pGL3–IκB-ζ (−853) together with pHβ-Tax and the indicated dominant negative mutants or empty vector. DN, dominant negative. The results are expressed as fold induction by Tax relative to the vector alone. (F) Cells were transfected with the indicated IκB-ζ reporter constructs together with pHβ-Tax, pcDNA3-RelA, or empty vector. The activities are expressed relative to that of cells transfected with pGL3–IκB-ζ (−853) and empty vector, which was defined as 1. The internal control reporter phRL-TK was cotransfected in all experiments. Data are means ± SD of three independent transfection experiments.

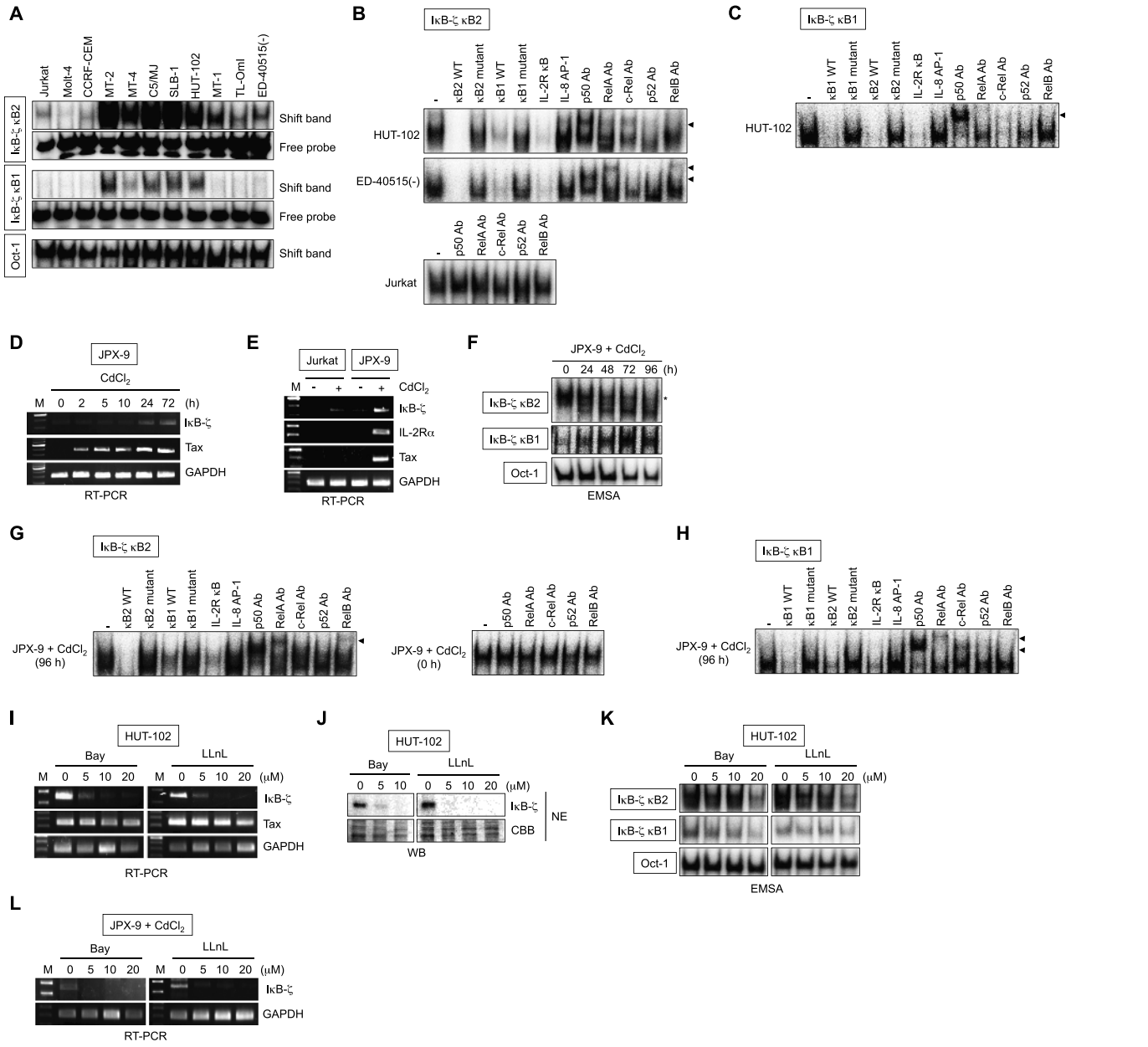

Figure 3. NF-κB subunits bound to two NF-κB–binding sites of the IκB-ζ promoter are essential for the Tax-induced *IκB-ζ* gene expression. (A) EMSA with the κB2- and κB1-binding sites of the IκB-ζ promoter in NEs from T cell lines. NEs from T cell lines were incubated with the indicated probes. Oct-1 regulates the transcription of a number of housekeeping genes. (B and C) Supershift assay and competition analysis with the κB2- and κB1-binding sites in T cell lines. NEs from HUT-102, ED-40515(−), and Jurkat cells were treated with the indicated Abs and incubated with κB2- or κB1-binding site probe in the absence or presence of the unlabeled oligonucleotides representing the IκB-ζ WT κB2 site, an IκB-ζ κB2 mutant site, the IκB-ζ WT κB1 site, an IκB-ζ κB1 mutant site, an NF-κB site from the IL-2Rα promoter (IL-2R κB), or an AP-1 site from the IL-8 promoter (IL-8 AP-1). Arrowhead, the DNA-binding complex supershifted by Ab. (D) Expression of IκB-ζ and Tax mRNA in $CdCl_2$-treated JPX-9 cells. JPX-9 cells were treated with $CdCl_2$ for the indicated time periods. RT-PCR analysis was carried out for IκB-ζ, Tax, and GAPDH (loading control). (E) Jurkat or JPX-9 cells were treated with or without $CdCl_2$ for 24 hours. RT-PCR analysis was carried out for IκB-ζ, IL-2Rα, Tax, and GAPDH (loading control). (F) EMSA with the κB2- and κB1-binding sites of the IκB-ζ promoter in NEs from $CdCl_2$-treated JPX-9 cells. Cells were treated with $CdCl_2$ for the indicated time periods. NEs were incubated with the indicated probes. *Nonspecific band. (G and H) Supershift assay and competition analysis with the κB2- and κB1-binding sites of the IκB-ζ promoter in $CdCl_2$-treated JPX-9 cells. Cells were treated with $CdCl_2$ for 96 hours. NEs from untreated (G, right) and $CdCl_2$-treated JPX-9 cells (G, left and H) were treated with the indicated Ab and incubated with κB2- or κB1-binding site probe in the absence or presence of the unlabeled indicated oligonucleotides. Arrowhead, the DNA-binding complex supershifted by Ab. (I) HUT-102 cells were treated with NF-κB inhibitors, Bay or LLnL (0, 5, 10, or 20 μM), for 24 hours. RT-PCR analysis was carried out for IκB-ζ, Tax, and GAPDH (loading control). (J) Expression of IκB-ζ protein in NEs from Bay- or LLnL-treated HUT-102 cells. Western blot analysis for IκB-ζ was performed by immunoblot analysis with anti–IκB-ζ Ab. CBB staining was carried out for loading control. (K) EMSA with the κB2- and κB1-binding sites of the IκB-ζ promoter in NEs from Bay- or LLnL-treated HUT-102 cells. NEs were incubated with the indicated probes. (L) JPX-9 cells were pretreated with either Bay or LLnL (0, 5, 10, or 20 μM) for 1 hour before treatment with $CdCl_2$ for another 24 hours. RT-PCR analysis was carried out for IκB-ζ and GAPDH (loading control).

on the NF-κB site from the IL-2Rα promoter all efficiently competed with the labeled κB2 probe and eliminated the binding of NEs from HUT-102 and ED-40515(–) cells (Figure 3*B*). In addition, the unlabeled IκB-ζ κB1 and κB2 oligonucleotides or NF-κB site from the IL-2Rα promoter effectively competed with the labeled κB1 probe and eliminated the binding of NEs from HUT-102 cells (Figure 3*C*). In contrast, the unlabeled IκB-ζ κB2 and κB1 mutants or consensus AP-1 site from the IL-8 promoter could not compete with the labeled κB2 and κB1 probes (Figure 3, *B* and *C*). The composition of the transcription factor DNA-protein complexes in HUT-102 and ED-40515(–) cells was analyzed by supershift assay using specific Abs (Figure 3, *B* and *C*). These experiments indicated that the complexes κB2 and κB1 in HUT-102 corresponded to p50, RelA, c-Rel, and p52, and the complexes κB1 in ED-40515(–) corresponded to p50, RelA, c-Rel, and RelB. In contrast, DNA-protein complexes to the IκB-ζ κB2 site in Jurkat cells were not supershifted by any of the NF-κB subunit Abs, suggesting that protein complexes to the κB2 site detected in Jurkat cells do not consist of NF-κB proteins (Figure 3*B*, *bottom*). These results suggest that NF-κB components are bound to the κB1 and κB2 sites of the IκB-ζ promoter in HTLV-I–transformed T cell lines and to the κB1 site alone in ATL-derived T cell lines, respectively.

Tax Induces IκB-ζ Gene Expression through NF-κB Activation

Treatment of JPX-9 cells, a Jurkat subline that carries the *tax* gene under the control of the *metallothionein* gene promoter [14], with $CdCl_2$ rapidly induced Tax mRNA expression (Figure 3*D*). IκB-ζ mRNA was also induced by 24 hours after treatment with $CdCl_2$ (Figure 3*D*). In contrast, the same treatment did not induce Tax, IL-2Rα, and IκB-ζ mRNA expression in Jurkat cells (Figure 3*E*). In addition, EMSA analysis of NEs from the $CdCl_2$-treated JPX-9 cells showed that Tax expression alone elicited binding to the IκB-ζ κB2 and κB1 probes (Figure 3*F*). The unlabeled IκB-ζ κB2 and κB1 oligonucleotides or an NF-κB site from the IL-2Rα promoter effectively competed with the labeled κB2 and κB1 probes and eliminated the binding of NEs from $CdCl_2$-treated JPX-9 cells [Figure 3, *G* (*left*) and *H*]. In contrast, the unlabeled IκB-ζ κB2 and κB1 mutants or consensus AP-1 site could not compete with the labeled probes [Figure 3, *G* (*left*) and *H*]. Supershift assay demonstrated that complex κB2 corresponded to p50, RelA, and RelB, and complex κB1 corresponded to p50, RelA, and c-Rel [Figure 3, *G* (*left*) and *H*]. In contrast, the protein complexes bound to the κB2 oligonucleotide in untreated JPX-9 cells were not supershifted by any of the NF-κB subunit Abs (Figure 3*G*, *right*). These data suggest that Tax induces expression by activating the binding of NF-κB proteins to two NF-κB sites in the *IκB-ζ* gene promoter.

To determine the other roles of NF-κB in IκB-ζ expression, we used NF-κB signaling inhibitors. Both Bay 11-7082, an inhibitor of IκBα phosphorylation [48], and LLnL, a proteasomal inhibitor [49], inhibited the expression of IκB-ζ at the mRNA (Figure 3*I*) and protein (Figure 3*J*) levels and NF-κB binding to the IκB-ζ κB2 and κB1 probes in HUT-102 cells (Figure 3*K*). Both inhibitors suppressed the Tax-induced expression of IκB-ζ mRNA in $CdCl_2$-treated JPX-9 cells (Figure 3*L*). These findings indicate that the Tax-induced IκB-ζ expression is mediated mainly through the activation of NF-κB.

IκB-ζ Induces Bcl3 *Gene Expression in an NF-κB–Dependent Manner*

To analyze IκB-ζ–induced gene expression in T cells, we compared the gene expression profiles by cDNA microarray analysis using RNA isolated from Jurkat cells transfected with IκB-ζ or a control vector. In IκB-ζ–expressing Jurkat cells, 19 genes were upregulated (five-fold or greater) compared with control Jurkat cells (Table 3). One member of the IκB family, *Bcl3*, is known to be an NF-κB–regulatory gene similar to *IκB-ζ* [31]. In addition, the following genes are all interferon (IFN)–stimulated genes (ISGs): *GBP-1* [32]; *GBP-2*; *GBP-5*; *STAT1* [33]; *NLR family, CARD domain containing 5*; *epithelial stromal interaction 1 (breast)*; *4-aminobutyrate aminotransferase*; *transporter 1, ATP-binding cassette, subfamily B*; *regulator of G protein signaling 9*; *interferon-induced protein with tetratricopeptide repeats 3*; *interferon-induced protein 44-like*; *poly(ADP-ribose) polymerase family, member 9*; *Kelch domain containing 7B*; *interferon, α-inducible protein 27*. These results suggest that IκB-ζ induces the expression of NF-κB– and IFN-regulatory genes in T cells. Next, we analyzed the levels of some of these specific mRNAs by RT-PCR. IκB-ζ dose-dependently induced *Bcl3*, *GBP-1*, *STAT1*, and *SLAMF7* gene expression in Jurkat cells (Figure 4*A*).

To determine the effects of IκB-ζ on *Bcl3* gene expression at the transcriptional level, we performed luciferase reporter assays using Bcl3 promoter/enhancer–luciferase reporter plasmids [31]. IκB-ζ dose-dependently activated Bcl3 expression through the promoter region together with the intronic enhancer region 3 (Pr11 E3) in Jurkat cells (Figure 4*B*). Bcl3 E3 contains one NF-κB–binding sequence (Figure 4*C*), and IκB-ζ only modestly activated Bcl3 expression through the upstream promoter (Pr11) or the promoter region together with enhancer region 4 (Pr11 E4). IκB-ζ also activated Bcl3 transcription through the promoter containing both E3 and E4 (Pr11 E3 + E4; Figure 4*C*). To further confirm that the NF-κB pathway was responsible for IκB-ζ–induced expression of Bcl3, we tested a Bcl3 reporter construct carrying a mutation in the NF-κB site. Figure 4*C* shows that the NF-κB site in E3 is the primary element responsible for IκB-ζ induction of the *Bcl3* gene. However, previous studies have shown that Tax mediates Bcl3 induction through an intronic enhancer [50] and Bcl3 is constitutively expressed in HTLV-I–infected T cells [51]. For this reason, we investigated the synergistic effect of Tax and IκB-ζ on Bcl3 transactivation. Bcl3 Pr11 E3 responded to Tax and RelA, but Bcl3 Pr11 E3ΔκB reduced

Table 3. Genes Upregulated by IκB-ζ (Fold Change ≥ 5.0).

Gene Name	Gene Symbol	Accession No.	Fold Change
SLAM family member 7	*SLAMF7*	NM_021181	20.8
Guanylate-binding protein 1	*GBP-1*	NM_002053	12.1
Guanylate-binding protein 2	*GBP-2*	NM_004120	9.6
B cell CLL/lymphoma 3	*Bcl3*	NM_005178	9.3
NLR family, CARD domain containing 5	*NLRC5*	NM_032206	8.0
Guanylate-binding protein 5	*GBP-5*	NM_052942	8.0
Epithelial stromal interaction 1 (breast)	*EPSTI1*	NM_033255	8.0
Signal transducer and activator of transcription 1	*STAT1*	NM_139266	7.8
4-Aminobutyrate aminotransferase	*ABAT*	NM_000663	7.2
G protein–coupled receptor 18	*GPR18*	NM_005292	7.0
Transporter 1, ATP-binding cassette, subfamily B	*TAP1*	NM_000593	6.8
Regulator of G protein signaling 9	*RGS9*	NM_003835	6.8
Interferon-induced protein with tetratricopeptide repeats 3	*IFIT3*	NM_001549	6.5
Interferon-induced protein 44-like	*IFI44L*	NM_006820	6.4
Poly(ADP-ribose) polymerase family, member 9	*PARP9*	NM_031458	5.5
Tubulin polymerization promoting protein	*TPPP*	NM_007030	5.5
Interleukin-4–induced 1	*IL-4I1*	NM_172374	5.3
Kelch domain containing 7B	*KLHDC7B*	NM_138433	5.0
Interferon, α-inducible protein 27	*IFI27*	NM_005532	5.0

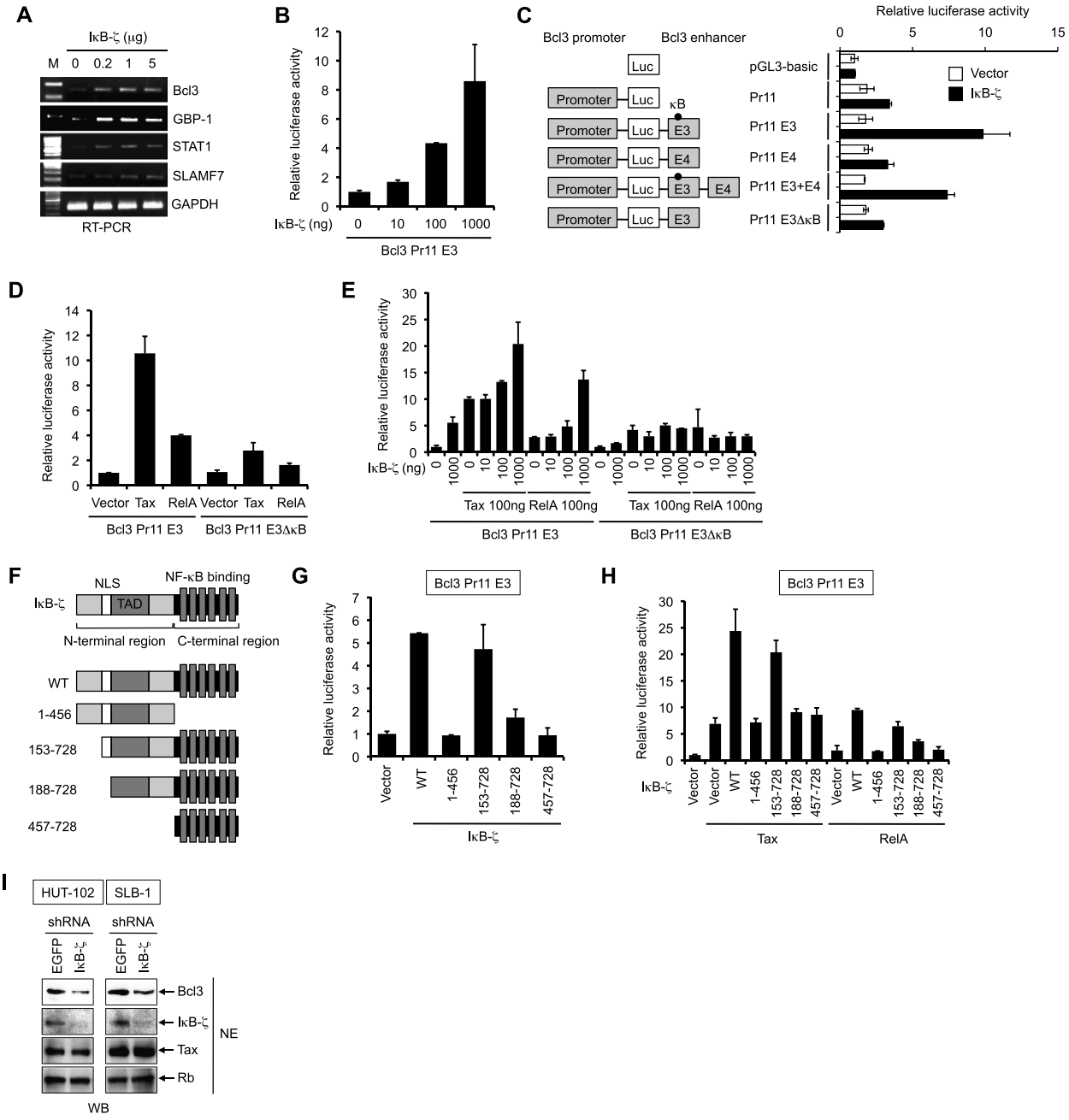

Figure 4. IκB-ζ and Tax activate *Bcl3* gene expression in an NF-κB–dependent manner. (A) Gene expression in IκB-ζ–expressing Jurkat cells. Cells were transfected with various amounts of pcDNA3–FLAG–IκB-ζ. Cells were harvested 24 hours post-transfection, and RT-PCR analysis was carried out for Bcl3, GBP-1, STAT1, SLAMF7, and GAPDH (loading control). (B) Jurkat cells were transfected with a luciferase reporter containing the Bcl3 promoter in combination with enhancer E3 (pGL3–Bcl3 Pr11 E3) together with the various amounts of pcDNA3–FLAG–IκB-ζ. Luciferase activity was measured 24 hours after transfection, and the results are expressed as fold induction by IκB-ζ relative to the vector alone. (C) Cells were transfected with the indicated reporter constructs containing the Bcl3 promoter either alone or in combination with enhancers E3, E4, and E3/E4 together with pcDNA3–FLAG–IκB-ζ or empty vector. The activities are expressed relative to that of cells transfected with pGL3-basic and empty vector, which was defined as 1. (D) Cells were transfected with pGL3–Bcl3 Pr11 E3 or pGL3–Bcl3 Pr11 E3ΔκB together with empty vector, pHβ-Tax, or pcDNA3-RelA. The activities are expressed relative to that of cells transfected with pGL3–Bcl3 Pr11 E3 and empty vector, which was defined as 1. (E) Cells were transfected with pGL3–Bcl3 Pr11 E3 or pGL3–Bcl3 Pr11 E3ΔκB together with the various amounts of pcDNA3–FLAG–IκB-ζ in the absence or presence of pHβ-Tax or pcDNA3-RelA. The activities are expressed relative to that of cells transfected with pGL3–Bcl3 Pr11 E3 and empty vector, which was defined as 1. (F) Schematic diagrams of WT IκB-ζ and IκB-ζ deletion mutants containing IκB-ζ amino acids 1 to 456, 153 to 728, 188 to 728, and 457 to 728. (G) Jurkat cells were transfected with pGL3–Bcl3 Pr11 E3 together with empty vector, pcDNA3–FLAG–WT IκB-ζ, or IκB-ζ deletion mutants. The results are expressed as fold induction by WT IκB-ζ or IκB-ζ mutants. (H) Cells were transfected with pGL3–Bcl3 Pr11 E3 with WT IκB-ζ or IκB-ζ mutants in the absence or presence of pHβ-Tax or pcDNA3-RelA. The activities are expressed relative to that of cells transfected with pGL3–Bcl3 Pr11 E3 and empty vector, which was defined as 1. The internal control reporter phRL-TK was cotransfected in all experiments. Data are means ± SD of three independent transfection experiments. (I) Expression of Bcl3 in NEs from IκB-ζ knockdown HUT-102 and SLB-1 cells. Western blot analysis for Bcl3, IκB-ζ, Tax, and Rb (loading control) was performed by immunoblot analysis.

the response to Tax and RelA (Figure 4*D*). In addition, IκB-ζ dose-dependently enhanced Tax- and RelA-induced Bcl3 Pr11 E3 activation, but it was not observed in Bcl3 Pr11 E3ΔκB (Figure 4*E*). These results suggest that IκB-ζ synergistically enhances Tax- and RelA-induced Bcl3 transactivation in an NF-κB–dependent manner.

Next, we investigated the domains of IκB-ζ required for Bcl3 transactivation. IκB-ζ is separable into two parts, amino (N)–terminal and carboxyl-terminal regions (Figure 4*F*) [7]. The N-terminal region of IκB-ζ contains a nuclear localization signal (NLS) and a transcriptional activation domain (TAD). The carboxyl-terminal region of IκB-ζ harbors the ankyrin repeats, which are responsible for NF-κB binding. We used full-length WT IκB-ζ– and IκB-ζ deletion mutant–expressing vectors containing IκB-ζ amino acids 1 to 456, 153 to 728, 188 to 728, and 457 to 728 (Figure 4*F*). The deletion mutants of IκB-ζ amino acids 1 to 456, 188 to 728, and 457 to 728 did not activate Bcl3 Pr11 E3, while WT IκB-ζ and the deletion mutant of IκB-ζ amino acids 153 to 728 induced Bcl3 Pr11 E3 transactivation (Figure 4*G*). In addition, WT IκB-ζ and IκB-ζ amino acids 153 to 728, but not 1 to 456, 188 to 728, and 457 to 728, enhanced Tax- and RelA-induced Bcl3 transcription (Figure 4*H*). These results highlight the importance of the TAD-, NLS-, and NF-κB–binding domains of IκB-ζ in Bcl3 transactivation.

To investigate the role of IκB-ζ in Bcl3 expression in HTLV-I–infected T cells, we performed knockdown of IκB-ζ in HUT-102 and SLB-1 cells using IκB-ζ shRNA (Figure 4*I*). Silencing IκB-ζ expression decreased Bcl3 expression but did not affect Tax expression levels in these cells.

IκB-ζ Induces Expression of IFN-Regulatory Genes, GBP-1, *and* STAT1

The genes upregulated by IκB-ζ included several IFN-regulatory genes. Therefore, we investigated the effect of IκB-ζ on the expression of two of these, *GBP-1* and *STAT1* genes at the transcriptional level. First, we performed luciferase reporter assays using several GBP-1 luciferase reporter plasmids. IκB-ζ, but not Tax, activated Pro3757–GBP-1 (position, −1778 to +1979), Pro1762–GBP-1 (−1778 to −17), and Pro237–GBP-1 (−218 to +19), indicating that Pro237–GBP-1 is sufficient to mediate GBP-1 expression in response to IκB-ζ (Figure 5*A*). Previous studies showed that the NF-κB motif and IFN-α–stimulated response element (ISRE) cooperate in the activation of GBP-1 expression by inflammatory cytokines [32]. To determine the role of the NF-κB and ISRE sites on GBP-1 expression induced by IκB-ζ, single or double mutants of the respective sites were used. IκB-ζ could not transactivate Pro237–GBP-1 ΔISRE or Pro237–GBP-1 ΔISRE/cRel mutants but activated the Pro237–GBP-1 ΔcRel mutant (Figure 5*A*). These data suggest that IκB-ζ induces GBP-1 transcription in an ISRE-dependent manner. We also investigated the role of IκB-ζ in GBP-1 expression in HTLV-I–infected T cells. Depletion

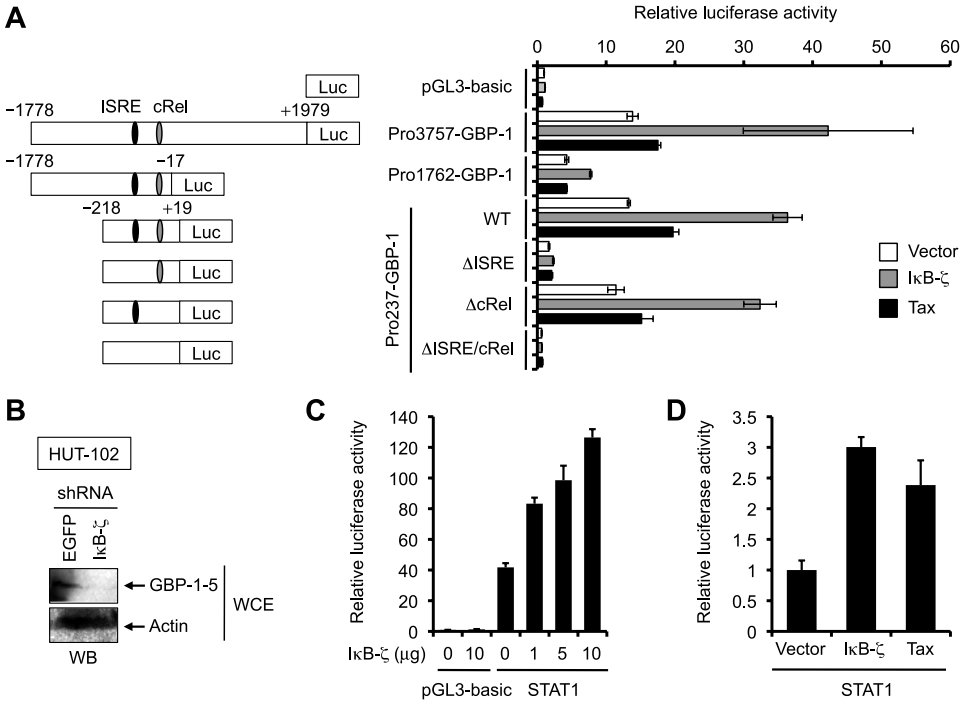

Figure 5. IκB-ζ activates *GBP-1* and *STAT1* gene transcription. (A) Jurkat cells were transfected with the indicated GBP-1 reporter constructs together with empty vector, pcDNA3–FLAG–IκB-ζ, or pHβ-Tax. The activities are expressed relative to that of cells transfected with pGL3-basic and empty vector, which was defined as 1. (B) Expression of GBP-1 in WCEs from IκB-ζ knockdown HUT-102 cells. Western blot analysis for GBP-1 to GBP-5 and actin (loading control) was performed by immunoblot analysis. (C) Jurkat cells were transfected with pGL3-basic or STAT1 promoter/enhancer reporter construct together with empty vector or various amounts of pcDNA3–FLAG–IκB-ζ. The activities are expressed relative to that of cells transfected with pGL3-basic and empty vector, which was defined as 1. (D) Cells were transfected with STAT1 promoter/enhancer reporter construct together with empty vector, pcDNA3–FLAG–IκB-ζ, or pHβ-Tax. Cells were harvested 24 hours post-transfection, and luciferase activity was measured. The results are expressed as fold induction by IκB-ζ or Tax relative to the vector alone. The internal control reporter phRL-TK was cotransfected in all experiments. Data are means ± SD of three independent transfection experiments.

of IκB-ζ decreased GBP-1 to GBP-5 expression in HUT-102 cells (Figure 5*B*).

Next, we studied the effect of IκB-ζ on *STAT1* gene expression at the transcriptional level by luciferase assays using STAT1 promoter/enhancer–luciferase reporter plasmid (position, −338 to +1033). IκB-ζ dose-dependently induced *STAT1* promoter/enhancer activation in Jurkat cells (Figure 5*C*). In addition, Tax transactivated the STAT1 promoter/enhancer similar to IκB-ζ. These data suggest that IκB-ζ and Tax induce STAT1 transactivation in T cells.

IκB-ζ Modulates Tax-Induced Gene Transactivation

IκB-ζ is known as a positive and negative regulator of NF-κB–mediated transcription [7,12,13]. Therefore, we investigated the effect of IκB-ζ on the expression of several genes known to be induced by either Tax or NF-κB. For example, previous studies showed that the promoters of *IL-8* [52] and *iNOS* genes [53] contain NF-κB–binding sequences and are also regulated by Tax and NF-κB [52–54]. IκB-ζ dose-dependently inhibited Tax-induced activation of IκB-ζ (−853; Figure 6*A*) and IL-8 −133 (Figure 6*B*) reporter plasmids in Jurkat cells, while IκB-ζ alone did not affect the activation of these reporter plasmids. In contrast, IκB-ζ dose-dependently activated the iNOS promoter and enhanced Tax-induced iNOS promoter activation (Figure 6*C*). In addition, analysis using an NF-κB reporter plasmid (NF-κB–Luc) indicated that IκB-ζ dose-dependently inhibited Tax- and RelA-induced NF-κB activation (Figure 6*D*). Furthermore, we attempted to identify the domain(s) of IκB-ζ required to inhibit Tax- and RelA-induced NF-κB activation. The deletion mutants of IκB-ζ amino acids 1 to 456 and 457 to 728 did not inhibit Tax-induced NF-κB activation. The deletion mutants of IκB-ζ amino acids 1 to 456, 153 to 728, and 457 to 728 did not show any inhibition of RelA-induced NF-κB activation, whereas IκB-ζ amino acids 188 to 728 exhibited moderate inhibition (Figure 6*E*). These results indicate that the N-terminal 187 amino acid and NLS are not required for the inhibition of Tax- and RelA-induced NF-κB activation, respectively.

Transcriptional induction of IL-8 and iNOS depends on cooperative activities of NF-κB and AP-1 [52,55]. AP-1 also plays a role in the viral transcription from the HTLV-I LTR [56]. IκB-ζ dose-dependently inhibited Tax-induced AP-1 and HTLV-I LTR activation (Figure 6, *F* and *H*). The deletion mutant of IκB-ζ amino acids 457 to 728 did not inhibit Tax-induced AP-1 activation, and the other mutants similarly did not show inhibitory activity, although their levels varied (Figure 6*G*). These results suggest that the N-terminal 456 amino acid, and to a lesser extent the carboxyl-terminus, are important for the inhibition of Tax-induced AP-1 activation. Taken together, IκB-ζ exhibits dual roles in Tax-mediated transcription.

IκB-ζ Interacts with Tax

Finally, immunoprecipitation with anti-Tax Ab demonstrated that IκB-ζ associated with Tax in 293T cells (Figure 7*A*). In addition, endogenous IκB-ζ associated with Tax in HUT-102 cells (Figure 7*B*). GST pull-down assay by GST-Tax showed that IκB-ζ physically interacted with Tax (Figure 7*C*). WT IκB-ζ and the deletion mutants of IκB-ζ amino acids 1 to 456, 153 to 728, and 188 to 728, but not the deletion mutant of IκB-ζ amino acids 457 to 728, interacted with Tax (Figure 7*D*). These data suggest that N-terminal IκB-ζ interacts with Tax.

Discussion

In this study, we showed Tax-dependent and Tax-independent *IκB-ζ* gene expression in HTLV-I–infected T cell lines and ATL cells and that such expression is NF-κB dependent. Our results are consistent with oligonucleotide microarray data showing that IκB-ζ is one of the genes that is upregulated more than 10-fold in three HTLV-I–infected T cell lines compared with an uninfected T cell line [57]. Our results also demonstrated that Tax stimulation of the NF-κB pathway is the primary route for IκB-ζ transcription, although CREB phosphorylation also seemed to be involved in this process. The promoter region, spanning positions −853 to −17, does not include CREB-binding sites. However, the Tax mutant 703 defective for CREB activation, repressed IκB-ζ promoter activation, and the disruption of CREB phosphorylation, by overexpressing CREB133, partially inhibited the promoter activity in response to Tax. Collectively, these findings indicate that although CREB phosphorylation is not essential, it contributes to full IκB-ζ promoter activation.

Tax-negative ATL-derived T cell lines expressed low levels of IκB-ζ mRNA and did not express the IκB-ζ protein compared with Tax-positive transformed T cell lines. In EMSA using extracts from ATL-derived T cell lines, oligonucleotide based on the *IκB-ζ* gene κB1 site showed no detectable binding activity and that of the κB2 site exhibited reduced binding activity compared with Tax-positive T cell lines. Thus, a correlation was noted between IκB-ζ expression levels and detection of NF-κB–binding activity. Furthermore, activation of the IκB-ζ promoter induced by Tax was almost eliminated by the deletion of the κB2 site. Taken together, it is clear that the κB2 site is the major critical target for IκB-ζ promoter activation.

In peripheral blood ATL cells, IκB-ζ mRNA levels did not correlate with those of Tax mRNA. However, immunohistochemical staining identified ATL cells positive for IκB-ζ in all lymph node samples examined. Constitutive high NF-κB activity has been demonstrated typically in ATL cells in which Tax expression is repressed *in vivo* [6]. Thus, although NF-κB activation appears necessary for the constitutive expression of IκB-ζ, transactivation by Tax is probably not the only mechanism for overexpression of IκB-ζ in ATL cells.

IκB-ζ induced *Bcl3* gene expression and enhanced Tax-dependent *Bcl3* gene induction. Previous studies showed that Tax induced Bcl3 in an NF-κB–dependent manner, and Bcl3 is constitutively expressed in HTLV-I–infected T cells and ATL cells [50,51]. Bcl3 is an ankyrin repeat–containing NF-κB–regulatory factor that is highly homologous to IκB-ζ [7]. Enhanced Bcl3 expression leads to increased cell survival, proliferation, and malignant potential [58–60]. Bcl3 is also implicated in the pathogenesis of many cancers commonly involving coactivation of NF-κB p50 by Bcl3 as the transforming event [61–64]. These reports and our study suggest that IκB-ζ may play an important role in ATL genesis by inducing Bcl3 expression in HTLV-I–infected T cells.

The N-terminal region of IκB-ζ interacted with Tax. In addition, the TAD, NLS, and NF-κB–binding domains of IκB-ζ were required for Tax-dependent Bcl3 transactivation. These results indicate that IκB-ζ–Tax interaction is important for enhancement of Tax-dependent *Bcl3* gene expression by IκB-ζ. Recently, the importance of ubiquitination and sumoylation on Tax activation of NF-κB has been investigated [65]. It is interesting whether posttranslational modifications of Tax influence its interaction with IκB-ζ. Previous studies also showed that Bcl3 interacts with Tax, and this interaction inhibits Tax-dependent HTLV-I LTR transactivation

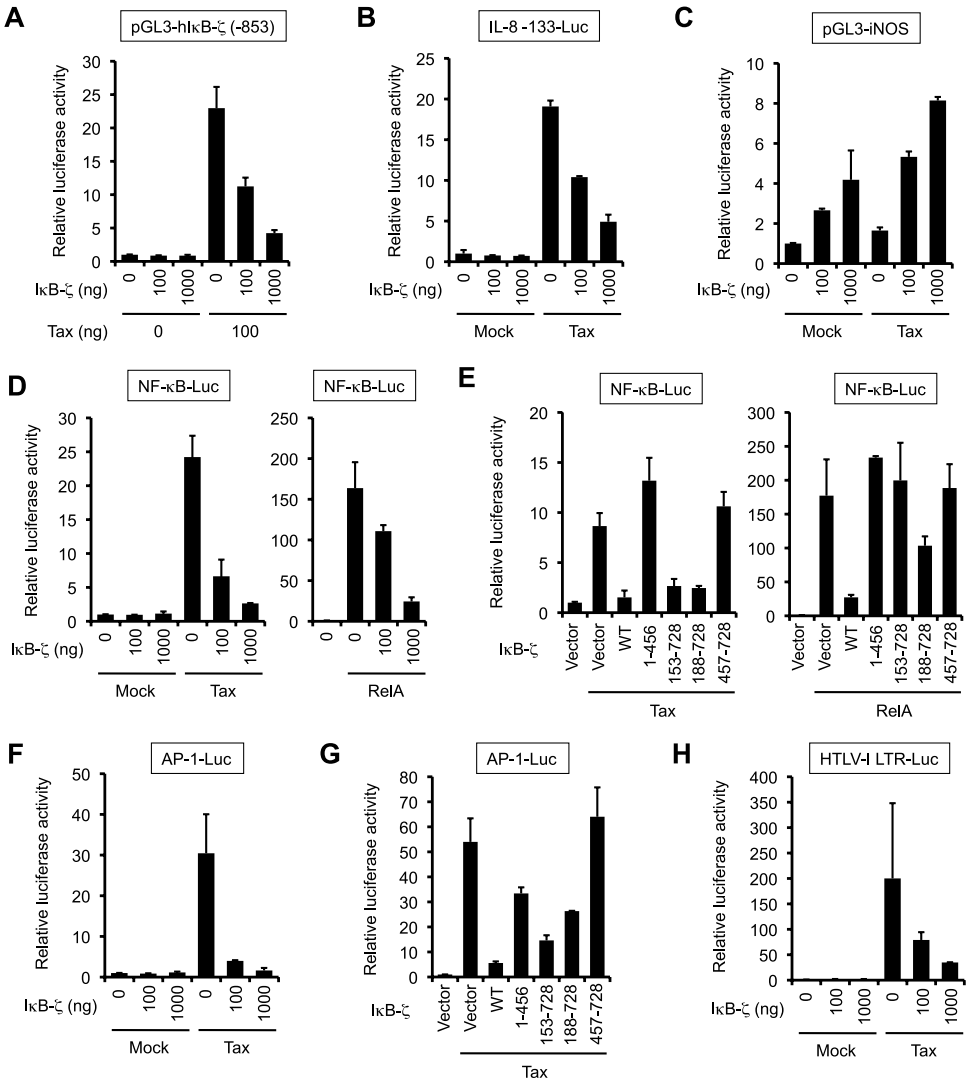

Figure 6. IκB-ζ modulates Tax-induced gene transactivation. Jurkat cells were transfected with (A) pGL3–hIκB-ζ (−853), (B) IL-8 −133-Luc, or (C) pGL3-iNOS together with the indicated amounts of empty vector or pcDNA3–FLAG–IκB-ζ in the absence or presence of pHβ-Tax. The activities are expressed relative to that of cells transfected with each reporter plasmid and empty vector, which was defined as 1. (D) Jurkat cells were transfected with NF-κB–Luc together with the indicated amounts of empty vector or pcDNA3–FLAG–IκB-ζ in the absence or presence of pHβ-Tax (left) or pcDNA3-RelA (right). The activities are expressed relative to that of cells transfected with NF-κB–Luc and empty vector, which was defined as 1. (E) Cells were transfected with NF-κB–Luc together with empty vector, pcDNA3–FLAG–IκB-ζ, or its deletion mutants in the absence or presence of pHβ-Tax (left) or pcDNA3-RelA (right). The activities are expressed relative to that of cells transfected with NF-κB–Luc and empty vector, which was defined as 1. (F) Cells were transfected with AP-1–Luc together with the indicated amounts of empty vector or pcDNA3–FLAG–IκB-ζ in the absence or presence of pHβ-Tax. The activities are expressed relative to that of cells transfected with AP-1–Luc and empty vector, which was defined as 1. (G) Cells were transfected with AP-1–Luc together with empty vector, pcDNA3–FLAG–IκB-ζ, or its deletion mutants in the absence or presence of pHβ-Tax. The activities are expressed relative to that of cells transfected with AP-1–Luc and empty vector, which was defined as 1. (H) Cells were transfected with HTLV-I LTR-Luc together with the indicated amounts of empty vector or pcDNA3–FLAG–IκB-ζ in the absence or presence of pHβ-Tax. Cells were harvested 24 hours post-transfection, and luciferase activity was measured. The activities are expressed relative to that of cells transfected with HTLV-I LTR–Luc and empty vector, which was defined as 1. The internal control reporter phRL-TK was cotransfected in all experiments. Data are means ± SD of three independent transfection experiments.

[50,66]. As shown in Figure 6*H*, IκB-ζ inhibited Tax-dependent LTR transactivation. These findings suggest that IκB-ζ and Bcl3 cooperatively inhibit the Tax-dependent HTLV-I transcription and viral replication in HTLV-I–infected T cells, resulting in facilitation of immune evasion.

Tax plays a central role in T cell transformation, but hyperactivation of Tax and NF-κB triggers cellular senescence and apoptotic responses [67,68]. We found that IκB-ζ inhibited Tax-mediated NF-κB activation (Figure 6, *D* and *E*), resulting in repression of some Tax-induced cellular gene expression. While these observations are seemingly paradoxical, our results demonstrate the pleiotropic roles of IκB-ζ in Tax-induced NF-κB activation and indicate that IκB-ζ can act to counterbalance the aberrant Tax-mediated NF-κB hyperactivation and, hence, may be established to play an important role in helping maintain proliferation and promote the survival of HTLV-I–infected T cells and ATL cells by preventing apoptosis.

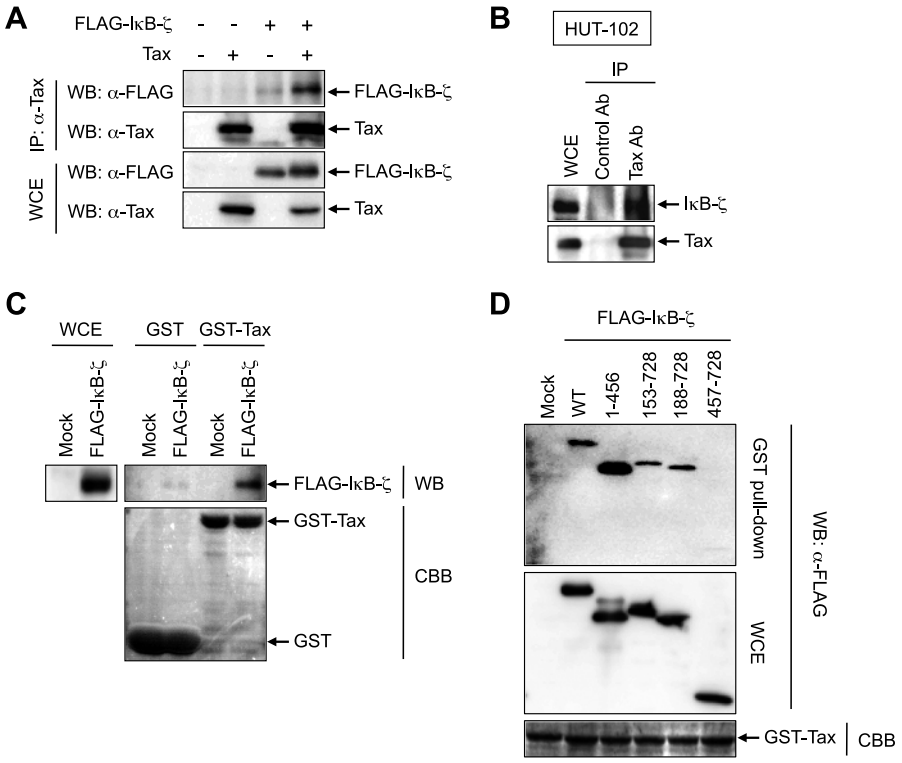

Figure 7. IκB-ζ interacts with Tax. (A) 293T cells were cotransfected with pcDNA3–FLAG–IκB-ζ and pHβ-Tax. Cells were harvested 24 hours post-transfection and lysed. WCEs were immunoprecipitated with anti-Tax Ab. Western blot analysis for FLAG–IκB-ζ and Tax was performed by immunoblot analysis with anti-FLAG and anti-Tax Abs, respectively. (B) WCEs from HUT-102 cells were immunoprecipitated with anti-Tax Ab, and Western blot analysis for IκB-ζ and Tax was performed by immunoblot analysis. (C) 293T cells were transfected with empty vector (Mock) or pcDNA3–FLAG–IκB-ζ. Cells were harvested 24 hours post-transfection and lysed. WCEs were mixed with glutathione sepharose 4B–conjugated GST (GST) or GST-Tax protein. The mixtures were incubated, washed, and eluted. The eluted complexes were subjected to Western blot analysis using anti-FLAG Ab. GST and GST-Tax were detected by CBB staining. (D) 293T cells were transfected with empty vector (Mock), pcDNA3–FLAG–WT IκB-ζ, or its deletion mutants. The lysates were incubated with GST-Tax fusion protein, pulled down, and analyzed by Western blot analysis using anti-FLAG Ab. Expression of FLAG–WT IκB-ζ or its deletion mutants were also performed by immunoblot analysis. GST-Tax was detected by CBB staining.

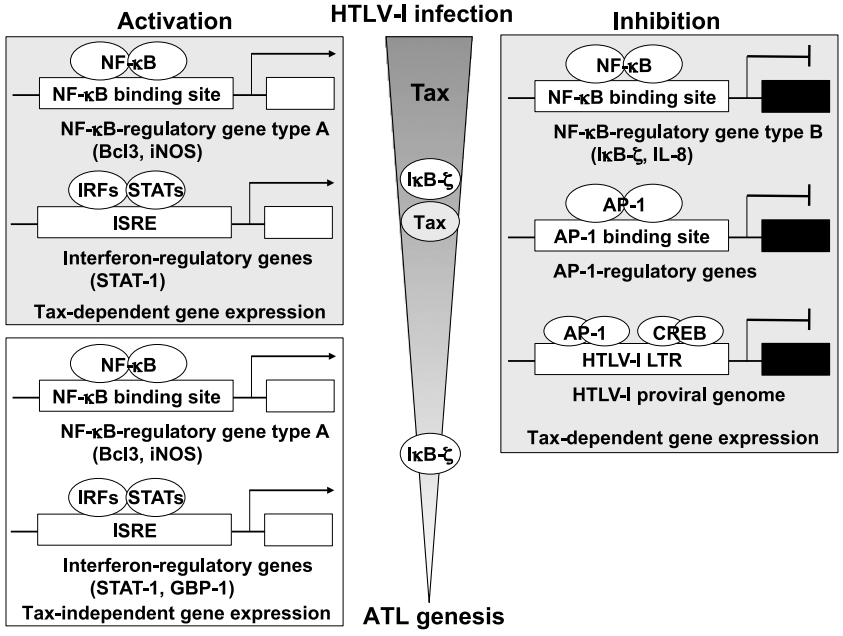

Figure 8. Pleiotropic roles of IκB-ζ in Tax-independent and Tax-dependent gene expression.

Surprisingly, IκB-ζ induced several ISGs in T cells. IκB-ζ, but not Tax, induced *GBP-1* gene expression in an ISRE-dependent manner. Consistent with our results, macrophages from IκB-ζ–deficient mice exhibited impairment of *GBP-1* gene expression in response to lipopolysaccharide [69]. GBP-1 enhances epidermal growth factor receptor–driven invasion in glioblastoma [70], and GBP-1 overexpression is associated with the resistant phenotype to the anticancer agent paclitaxel [71]. These findings suggest that induction of GBP-1 by IκB-ζ may be associated with malignant progression of ATL. In addition, IκB-ζ and Tax induced the expression of a central mediator of ISG expression, the *STAT1* gene in T cells. Previous reports have also shown that HTLV-I–transformed T cell lines and ATL cells express high levels of STAT1, and Tax induces *STAT1* gene expression in T cells [72,73]. However, the role of this STAT1/ISG axis in leukemogenesis remains to be clarified.

Figure 8 summarizes the findings of this study. In this model, IκB-ζ can bind to and modulate Tax function. IκB-ζ inhibits Tax-dependent IκB-ζ and IL-8 transactivation, whereas it induces and enhances Bcl3 and iNOS transactivation together with Tax in an NF-κB–dependent manner. Thus, IκB-ζ exhibits dual roles in NF-κB–mediated transcription in the presence of Tax, although the mechanism that determines the opposing actions of IκB-ζ on different genes remains to be defined. In addition to NF-κB–binding sites, another element may be required for the IκB-ζ–mediated transcriptional activation [74], because IκB-ζ acts as a negative regulator on the promoter harboring five repeats of NF-κB sequences (NF-κB–Luc [36]). IκB-ζ mutant, 188 to 728, deleted N-terminal 187 amino acid, failed to enhance Tax-induced Bcl3 transcription but inhibited Tax-induced NF-κB activation, suggesting that N-terminal 187 amino acid is important for enhancement of Tax-induced NF-κB activation but not required for inhibition of Tax-induced NF-κB activation. This region also interacts with Tax. IκB-ζ inhibited the activation of NF-κB by Tax and RelA. Similarly, the pleiotropic roles of Bcl3 in Tax-induced NF-κB activation have been recently reported [75]. In addition, IκB-ζ inhibited Tax-induced AP-1 and HTLV-I LTR activation. Tax induces IκB-ζ overexpression by activating the NF-κB pathway, and the elevated IκB-ζ might lead to increased cell proliferation, cell survival, and malignant potential by inducing expression of Bcl3, iNOS, GBP-1, and so on. We found that IκB-ζ negatively modulates Tax-induced NF-κB, AP-1, and HTLV-I LTR activation. It is likely that the Tax–IκB-ζ interaction at protein and gene expression levels results in a fine balance that ultimately endows a net evolutionary benefit to the survival of viral infected T cells. Furthermore, IκB-ζ may contribute to the pathogenesis of ATL, in which leukemic cells do not express Tax protein, through the up-regulation of NF-κB–regulated genes and ISGs.

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