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Tax-Independent Constitutive IκB Kinase Activation in Adult T-Cell Leukemia Cells[1]

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Abstract

Adult T-cell leukemia (ATL) is a fatal T-cell malignancy that arises long after infection with human T-cell leukemia virus type I (HTLV-I). We reported previously that nuclear factor-κB (NF-κB) was constitutively activated in ATL cells, although expression of the viral proteins was barely detectable, including Tax, which was known to persistently activate NF-κB. Here we demonstrate that ATL cells that do not express detectable Tax protein exhibit constitutive IκB kinase (IKK) activity. Transfection studies revealed that a dominant-negative form of IKK1, and not of IKK2 or NF-κB essential modulator (NEMO), suppressed constitutive NF-κB activity in ATL cells. This IKK activity was accompanied by elevated expression of p52, suggesting that the recently described noncanonical pathway of NF-κB activation operates in ATL cells. We finally show that specific inhibition of NF-κB by a super-repressor form of IκBα (SR-IκBα) in HTLV-I–infected T cells results in cell death regardless of Tax expression, providing definitive evidence of an essential role for NF-κB in the survival of ATL cells. In conclusion, the IKK complex is constitutively activated in ATL cells through a cellular mechanism distinct from that of Tax-mediated IKK activation. Further elucidation of this cellular mechanism should contribute to establishing a rationale for treatment of ATL.

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Keywords: ATL, leukemogenesis, NF-κB, IKK, NFKB2.

Introduction

Human T-cell leukemia virus type I (HTLV-I) is etiologically associated with the development of an aggressive and fatal malignancy of $CD4^+$ T lymphocytes called adult T-cell leukemia (ATL) and a variety of inflammatory disorders, including HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP), arthropathy, alveolitis, myositis, and uveitis [1–9]. Because there is a long latency period until the onset of diseases and a relatively low incidence of developing ATL after HTLV-I infection, leukemogenesis by HTLV-I has been thought to represent a multistep process [10].

Given its well-characterized oncogenic potential, Tax remains the focus of efforts to understand the mechanism by which HTLV-I transforms human T lymphocytes. Tax transactivates not only the HTLV-I long terminal repeat (LTR), but also transactivates or transrepresses the expression or functions of a wide array of cellular genes, including those for cytokines [11,12] and regulators of the cell cycle [13,14], DNA repair [15], or apoptosis [16,17]. Aberrant expression of these growth-related genes has been supposed to contribute to the establishment of the HTLV-I–associated proliferative disorders. Indeed, when expressed ectopically, Tax-immortalized primary human T cells and expression of Tax in rodent fibroblast cell lines results in their oncogenic transformation [18,19]. However, peripheral blood lymphocytes freshly isolated from ATL patients were reported to express the viral proteins at very low levels, if any [20–22]. In addition, nonsense or missense mutations of the tax gene were reported in certain ATL cases [23,24]. The lack of detectable viral gene expression in ATL cells suggests that the viral proteins, including Tax, are not necessary at the late stage of the disease and that a Tax-independent mechanism supports the gene expression for proliferation of leukemic cells. Studies in several ATL-derived cell lines and freshly isolated peripheral ATL cells identified constitutive nuclear factor-κB (NF-κB) activity as their common feature, suggesting a role for NF-κB in the ATL pathogenesis [25]. In line with this idea, inhibition of NF-κB activity by antisense oligonucleotides to RelA/p65 in Tax-transformed fibroblasts led to suppression of growth and impaired tumorigenicity in mice [26].

Abbreviations: ATL, adult T-cell leukemia; EMSA, electrophoretic mobility shift assay; mEcoVR, murine ecotropic viral receptor; β-gal, β-galactosidase; GST, glutathione-*S*-transferase; GFP, green fluorescent protein; HTLV-I, human T-cell leukemia virus type I; IκB, inhibitor of NF-κB; IKK, IκB kinase; LTR, long terminal repeat; Luc, luciferase; NF-κB, nuclear factor-κB; NEMO, NF-κB essential modulator; SRE, serum response element; SR-IκBα, super-repressor form of IκBα

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We most recently reported an inhibitor of NF-κB, Bay 11-7082, as being effective in inducing apoptosis of HTLV-I–transformed T-cell lines and primary ATL cells [27]. However, the low efficiencies of gene transduction to these cells have hindered a formal demonstration that specific inhibition of NF-κB through expression of an IκB-related protein induces cell death.

Transcription factor NF-κB is composed of dimeric complexes of the Rel/NF-κB family proteins. In mammals, these are RelA, cRel, RelB, p50, and p52. Among them, p50 and p52 are generated from the N-terminal portions of the precursor proteins NFKB1/p105 and NFKB2/p100, respectively. NF-κB activity is normally regulated tightly through its cytoplasmic retention by physical interaction with specific inhibitor proteins called IκB. This interaction masks the nuclear localization signal of NF-κB, preventing its nuclear translocation. A variety of stimuli lead to the phosphorylation of IκB proteins on conserved two serine residues (Ser-32 and Ser-36 on IκBα; Ser-19 and Ser-23 on IκBβ) by the IκB kinase (IKK) complex, which is comprised of two catalytic subunits, IKK1/α and IKK2/β, and a scaffolding protein NF-κB essential modulator (NEMO) [28–30]. This phosphorylation targets IκB for ubiquitination and subsequent proteosome-mediated degradation, resulting in the release of NF-κB. NF-κB then translocates to the nucleus where it binds to specific κB sites and modulates transcription. Most of the inducible NF-κB responses are mediated by the classical NF-κB heterodimers p50-RelA and are of transient nature mainly due to the rapid resynthesis of IκB proteins, representing the canonical pathway of NF-κB activation. This process requires IKK2 and NEMO. By contrast, the noncanonical pathway of NF-κB activation has recently been described, in which IKK1 controls the phosphorylation-dependent processing of NFKB2/p100 and generation of p52/RelB dimers [31,32]. Target genes activated by NF-κB include factors involved in apoptosis resistance, cell activation, and proliferation, as well as cytokines and chemokines involved in immune regulation [33]. Accumulating evidence has linked deregulated NF-κB activity to the pathogenesis of various cancers and hematopoietic malignancies, in which persistent NF-κB activity results from constitutive activation of the IKK signaling pathway or from dysfunction of IκB proteins [34,35]. In lymphoid malignancies, for example, NF-κB deregulation may occur due to chromosomal translocations in the *nfkb2* locus that result in constitutive processing of p100 [35]. It is reported that, unlike p50, production of p52 through a cotranslational mechanism is extremely poor, and that p100 does not undergo inducible processing in response to various stimuli [36,37]. Thus, p100 is expressed as its unprocessed form in most cases and acts as an IκB protein through the C-terminal ankyrin domains. Recent reports have demonstrated that IKK1, on activation by NIK or Tax, phosphorylates two specific serine residues in the C-terminus of p100, leading to its ubiquitination and subsequent generation of p52 [38–40]. However, it is largely unknown how ATL cells persistently activate NF-κB in the absence of Tax.

This report first demonstrates distinct features of IKK activation in ATL cells and T cells transformed *in vitro* by HTLV-I. Second, we show that Tax-independent NF-κB activation in ATL cells involves aberrant expression of the processed form of NFKB2. Finally, we provide formal evidence of the importance of NF-κB in the survival of ATL cells by showing that expression of a super-repressor form of IκBα (SR-IκBα) induces drastic cell death.

Materials and Methods

Cells

Jurkat [41] and Molt-4 [42] are HTLV-I–free human T-cell lines. MT-1 [43], TL-Om1 [44], and ED40515 (−) previously referred to as ED515-I [45] are HTLV-I–infected cell lines of leukemic cell origin. MT-2 [46], SLB-1 [47], MT-4 [48], and M8166 [49], a subclone of C8166 [50], are human T-cell lines transformed *in vitro* with HTLV-I. These T-cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Mouse ecotropic and amphotropic virus packaging cell lines, PLAT-E [51] and PLAT-A (T. Kitamura et al., unpublished), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate.

Transfection and Luciferase (Luc) Assay

Transient transfection of suspended cells was performed by a diethylaminoethyldextran (DEAE–dextran) procedure. 293T cells were transfected by a calcium phosphate coprecipitation method. The total amount of DNA transfected was always kept constant, and each transfection was internally controlled by cotransfection with a β-galactosidase (β-gal) expression plasmid (pEF1-LacZ). Assays for Luc and β-gal activities were performed 40 hours after transfection in standard methods. Luc activity was normalized on the basis of β-gal activity. Each experiment was repeated at least three times, and the results are expressed as a mean ± SD.

Plasmids

ConA-Luc [52], Igκ-ConA-Luc [52], EF1-lacZ (a kind gift of Dr. Memet; Institut Pasteur, Paris, France) [53], pcDNA3-dN97 [54], pRc-CMV-IκBαSR [55], and pCMV-Neo-Bam [56] were described previously. SRE-Luc reporter plasmid has three copies of serum response element (SRE) cloned in pGL vector (Promega, Madison, WI) and was kindly provided by Dr. Courtois (Institut Pasteur). pCMV-Neo-Bam-Tax was constructed by subcloning the *tax* gene from pH2R*wtax* [56] in pCMV-Neo-Bam. pMX-mEcoVR-puro was constructed by ligating a 2.3-kb *Eco*RI/*Bam*HI fragment of a pM5neo vector harboring the murine ecotropic viral receptor (*mEcoVR*) gene [57] with a 5.5-kb *Eco*RI/*Bam*HI fragment of pMX-puro [58]. An *Sca*I/*Bgl*II DNA fragment containing the cytomegalovirus early enhancer-promoter, U3-deleted murine leukemia virus LTR and packaging signal was excised from pRxhCD25iN [59], and subcloned into the same enzymatic sites of pMX-IRES-GFP [60], generating pMRX-IRES-GFP. Then, an *Eco*RI fragment of SR-IκBα from pRc-CMV-IκBαSR was subcloned in pMRX-IRES-GFP,

generating pMRX-SR-IκBα-GFP. VSV-G epitope-tagged dominant-negative IKK1 and IKK2 were generated by site-directed mutagenesis substituting Asn for Asp145 of pcDNA3GVSV-IKK1 [53] and pcDNA3GVSV-IKK2 [53]. The resultant plasmids were designated as pcDNA3GVSV-IKK1DN and pcDNA3GVSV-IKK2DN, respectively.

Antibodies

Anti–IκBα [61], anti–IκBβ [61], anti–Tax antibody (MI73) [62], and anti–NEMO [53] sera were described previously. Anti–IKK1 antibody (H-744), anti–IKK2 antibody (H-470), and anti–p52 antibody (C-5) and antiactin (C-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–IKK1 monoclonal antibody (B78-1) and anti–NEMO monoclonal antibody (C73-1794) were purchased from Becton Dickinson Pharmingen (San Diego, CA). Phospho-IκBα (Ser 32) antibody (9241) was purchased from Cell Signaling Technology (Beverly, MA).

Preparation of Cell Extracts

Cell fractionation was performed as described previously [63]. Briefly, exponentially growing cells left untreated or treated with 10 ng/ml tumor necrosis factor-α (TNFα) were suspended in buffer A containing 20 mM HEPES (pH 7.8), 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin. After cells were swollen on ice for 10 minutes, NP-40 was added to 1%. After vortex mixing for 15 seconds, the lysate was cleared of nuclei by centrifugation at 12,000 *g* at 4°C and the supernatant was used as cytoplasmic extract. The pelleted nuclei were washed with buffer B (20 mM HEPES [pH 7.8], 100 mM NaCl, 0.1 mM EDTA, and 25% glycerol), resuspended in appropriate volume of buffer C (20 mM HEPES [pH 7.8], 400 mM NaCl, 0.1 mM EDTA, 25% glycerol, 1 mM dithiothreitol [DTT], 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), vortexed vigorously, and agitated at 4°C for at least 20 minutes. Debris was removed by centrifugion at 12,000 *g* for 2 minutes and the supernatant was used as nuclear extract. For preparation of whole cell extract, cells were lysed in RIPA buffer (20 mM Tris–HCl [pH 8.0], 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.1 μM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin).

Western Blot Analysis

Cytoplasmic extracts or whole cell extracts were fractionated on SDS polyacrylamide gels and transferred onto Immobilon P membranes (Millipore, Billrica, MA). Blots were revealed with an enhanced chemiluminescence detection system (Perkin Elmer, Boston, MA).

Electrophoretic Mobility Shift Assay (EMSA)

Two micrograms of nuclear extracts was added to 15 μl of binding buffer (10 mM HEPES [pH 7.8], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 2.5% glycerol, 1 μg of poly [dI–dC]) and 0.5 ng of ^{32}P-labeled wild-type (KBF1) or mutated (KBFm) κB probe derived from the H-2K^b promoter [64] and incubated for 30 minutes at room temperature. Samples were run on a polyacrylamide gel containing 2.5% glycerol in 0.5 × TBE. Oligonucleotide containing the Octamer-binding site [65] was used as a nonspecific competitor for NF-κB binding.

Immunoprecipitation and Kinase Assay

Cytoplasmic extracts prepared from equivalent numbers of cells were subjected to immunoprecipitation with anti–NEMO serum or anti–IKK1 monoclonal antibody in TNT buffer (20 mM Tris–HCl [pH 7.5], 200 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM Na_3VO_4, and 20 mM β-glycerophosphate). Immunoprecipitates were collected on Protein G—Sepharose beads (Pierce, Boston, MA) which were then washed three times with TNT buffer and three times with kinase reaction buffer (20 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 100 μM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM DTT, and 20 μM ATP). Kinase reactions were performed for 30 minutes at 30°C using 5 μCi of [γ-^{32}P]ATP and glutathione-*S*-transferase (GST)-IκBα (amino acids 1–72) or GST-IκBβ (amino acids 1–56) as substrates. The reaction products were separated on 12% SDS polyacrylamide gels and revealed by autoradiography. The intensity of each band was determined by computerized image analysis. IKK activities of primary ATL cells were determined with *in vitro* kinase assay kit (Clontech, Palo Alto, CA).

Virus Infection and Cell Sorting

PLAT-A cells were transfected with pM5neo vector harboring the mEcoVR or pMXmEcoVR-puro by calcium phosphate precipitation, and the cell-free supernatant was used to transduce the *mEcoVR* gene to Jurkat, MT-1, and MT-2 cells. For infection, cells were exposed to viral supernatant for 2 hours at 37°C in the presence of 10 μg/ml polybrene. After G418 (0.5 mg/ml) or puromycin (1 μg/ml) selection, Jurkat and MT-1 cell clones and a pool of MT-2 cells expressing mEcoVR were established. Ecotropic retroviruses capable of expressing green fluorescent protein (GFP) alone, or SR-IκBα together with GFP, were prepared in PLAT-E cells and used for infection of cells stably expressing mEcoVR. At 72 hours postinfection, GFP-positive cells were sorted by FACSVantage flow cytometer (BD Biosciences, San Jose, CA). To assess the expression of SR-IκBα and NF-κB DNA-binding activity in sorted cells, cells were harvested and lysed at 80 hours postinfection for MT-2 cells and at 96 hours postinfection for Jurkat and MT-1 cells. Nuclear morphology of sorted cells stained with 10 μM Hoechst33342 was observed under a conforcal ultraviolet (UV) microscope at 84 hours postinfection for MT-2 cells and at 108 hours postinfection for the others. Cell viability was determined by trypan blue staining.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared with RNeasy Kit (Qiagen, Hilden, Germany) and the minus-strand cDNA were synthesized with Superscript first-strand synthesis RT-PCR

system (Invitrogen) using random hexamer primers. These cDNA were used as templates to amplify a region of HTLV-I *pX* using primers tax-4 (5′-ctgctctcatcccggtaagc-3′) and tax-7474 (5′-gagccgataacggctccatcga-3′). As an internal control, expression of β-*actin* mRNA was examined using primers BA3 (5′-aagagaggcatcctcaccct-3′) and BA2 (5′-tacatggctggggtgttgaa-3′) [66]. The resulting PCR products were analyzed by 5% polyacrylamide gel electrophoresis.

Results

Tax-Independent NF-κB Activation in ATL Cells

Previous studies have demonstrated that either freshly isolated leukemic cells or cell lines of leukemic cell origin do not express detectable viral proteins including Tax, but show constitutive NF-κB activity. Indeed, three HTLV-I–infected cell lines of leukemic cell origin used in this study (MT-1, ED40515 (−), and TL-Om1), but not those established through *in vitro* transformation by HTLV-I (MT-2, SLB-1, MT-4, and M8166), lack detectable expression of the Tax protein and its mRNA (Figure 1*A*). EMSA revealed elevated levels of DNA-binding activity to the oligonucleotide-containing κB site in these ATL cells as well as in Tax-positive HTLV-I–transformed cells, but not in HTLV-I–free Jurkat or Molt-4 cells (Figure 1*B*, *left upper panel*). Nuclear extract prepared from MT-1 cells did not give rise to a retarded band with labeled oligonucleotide probe containing mutated κB site. The DNA-binding activity in MT-1 cells was efficiently competed with an excess amount of cold probe containing wild-type κB site, but not with an excess amount of cold probe containing mutated κB site or Octamer-binding site (Figure 1*B*, *right panel*). These results demonstrate NF-κB–specific DNA-binding activity in Tax-positive and Tax-negative HTLV-I–infected cells. Western blot analyses detected IκBα and IκBβ in all tested cells (Figure 1*B*, *left lower panels*). This suggests that loss of IκB expression was not the cause of constitutive NF-κB activity in ATL cells. Very small amounts of IκBα detected in MT-4 and M8166 cells are likely due to relatively poor production and Tax-induced degradation of this molecule. IκBα phosphorylated at serine 32 was detected in all Tax-positive HTLV-I–transformed cells, although very weakly in MT-4 cells (Figure 1*C*). Moreover, the IκBα protein phosphorylated at serine 32 was detected in all tested ATL cells as well as in Jurkat cells stimulated with TNFα. This suggests elevated phosphorylation activity of IKK on this specific serine residue in ATL cells.

IKK1, But Not IKK2 or NEMO, Is Important for NF-κB Activation in ATL Cells

Steady-state levels of individual subunits of the IKK complex, IKK1, IKK2, and NEMO are shown in Figure 2*A*. Control T cells and ATL cells expressed similar amounts of IKK1. IKK1 expression was relatively poor in Tax-positive HTLV-I–transformed cells as reported previously [67]. There was no significant difference in the levels of IKK2 expression among all the tested cells, but more slowly migrating species of IKK2 were found in Tax-positive HTLV-I–transformed cells. Consistent with a previous report [68], treatment of cell extracts with alkaline phosphatase revealed that this slower migration represented phosphorylation events (data not shown). NEMO was detected in all the examined cells at the expected position, although its expression was relatively poor in Tax-positive HTLV-I–transformed cells.

To investigate the role for IKK in the constitutive activation of NF-κB in ATL cells, we transfected ATL and HTLV-I–transformed cells with a catalytically inactive form of IKK1 or IKK2 (dnIKK1 or dnIKK2) that functions as a dominant-negative inhibitor specific for NF-κB activation. When expressed in 293T cells, dnIKK1 and dnIKK2 efficiently suppressed Tax-induced NF-κB–dependent transcription, but did not interfere with Tax-induced HTLV-I LTR–directed transcription (Figure 2*B*). Besides, expression of these constructs did not suppress SRE-dependent transcription in MT-1 or TL-Om1 cells (Figure 2*B*), thus establishing the specificity of action of these molecules. NF-κB–specific reporter gene activation was demonstrated by cotransfecting each cell line with the vector plasmid and either ConA-Luc devoid of κB sites or Igκ-ConA-Luc (compare the left two columns in each panel of Figure 2*C*). These experiments revealed that the net NF-κB–dependent Luc activity in each cell line was robust, so that the Igκ-ConA-Luc reporter plasmid was used in the following experiments. Transient expression of dnIKK2 potently suppressed NF-κB–dependent transcription in MT-2 (88 ± 3%) and M8166 (47 ± 13%) cells, whereas it was virtually ineffective in ATL cells (MT-1 and TL-Om1). This is not due to poor expression of dnIKK2 in ATL cells because NF-κB activation following transfection of these cells with Tax was efficiently suppressed by cotransfection with dnIKK2 (Figure 2*D*). These results also suggest that NF-κB activity in ATL cells does not depend on Tax. However, expression of dnIKK1 suppressed NF-κB–dependent transcription most remarkably in MT-2 cells, and moderately in ATL cells (28 ± 11% for MT-1 and 23 ± 1% for TL-Om1 cells) to degrees similar to that for Tax-positive M8166 cells (25 ± 9%) (Figure 2*C*). The suppression of NF-κB–dependent transcription by dnIKK1 in these cells was statistically significant ($P<.05$). We also tested a dominant-negative form of NEMO (dN97) that had previously been shown to suppress TNFα-induced NF-κB activation [54]. Consistent with our genetic evidence that NEMO is essential for Tax-mediated NF-κB activation [53], expression of dN97 potently suppressed NF-κB activity in Tax-positive HTLV-I–transformed cells. In contrast, dN97 did not reduce NF-κB activity in ATL cells. Expression of a SR-IκBα that cannot be phosphorylated at the specific serine residues targeted by IKK efficiently reduced NF-κB activity in both types of HTLV-I–infected cells. Relatively higher expression of NEMO in ATL cells (Figure 2*A*) is unlikely to counteract the dominant-negative effect of dN97 because coexpression of dN97 and Tax in MT-1 and

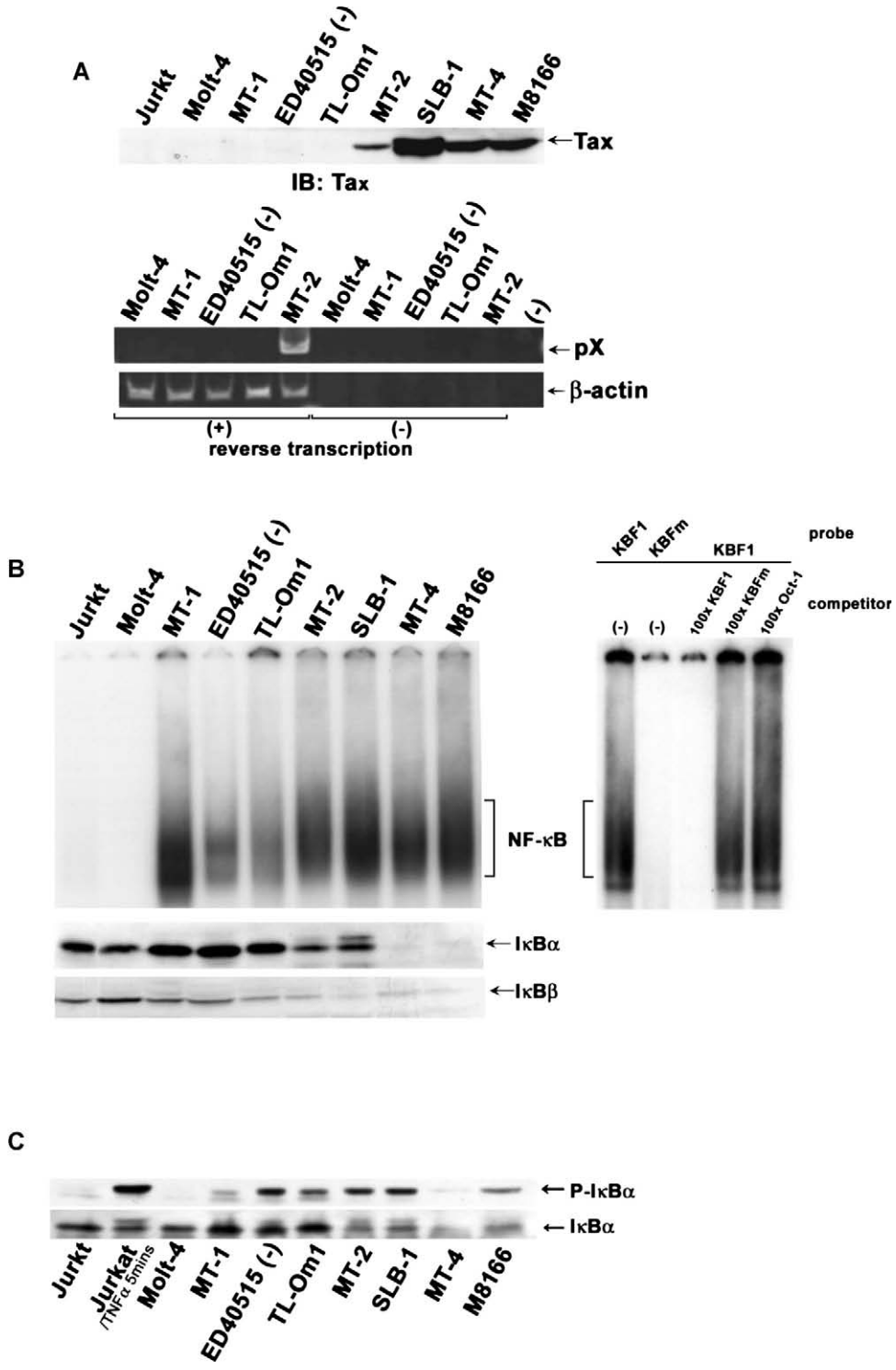

Figure 1. *Constitutive NF-κB activity in Tax-negative ATL cells. (A) Steady-state levels of Tax expression in equivalent numbers of cells were determined by Western blotting using a specific antibody MI73 (upper panel). Total RNA (1 μg) prepared from the indicated cells was subjected to RT-PCR. To determine the relative quantities of Tax and β-actin mRNA, cDNA were amplified using primers for HTLV-I pX with 35 cycles or those for β-actin with 18 cycles (lower panel). (B) Left upper panel: Two micrograms of nuclear extracts from the indicated cell lines was analyzed by EMSA using a labeled κB site oligonucleotide derived from the H-2K^b promoter (KBF1) as a probe. Right panel: EMSA was performed with 2 μg of nuclear extract from MT-1 cell using labeled wild-type (KBF1) or mutated (KBFm) κB oligonucleotides. A 100-fold excess of cold probe containing wild-type or mutated κB site, or probe containing Oct-1 site was added to show the specificity of binding. Left lower panels: Steady-state levels of IκBα and IκBβ in equivalent numbers of cells were determined by Western blotting using specific antibodies. (C) Using 50 μg of cytoplasmic extracts from Jurkat cells with or without 5 minutes of TNFα stimulation, Molt-4, and the indicated HTLV-I–infected cells, phosphorylation of IκBα was analyzed by Western blotting with a phospho-IκBα (Ser 32)—specific antibody, and the same membrane was reprobed with an IκBα-specific antibody.*

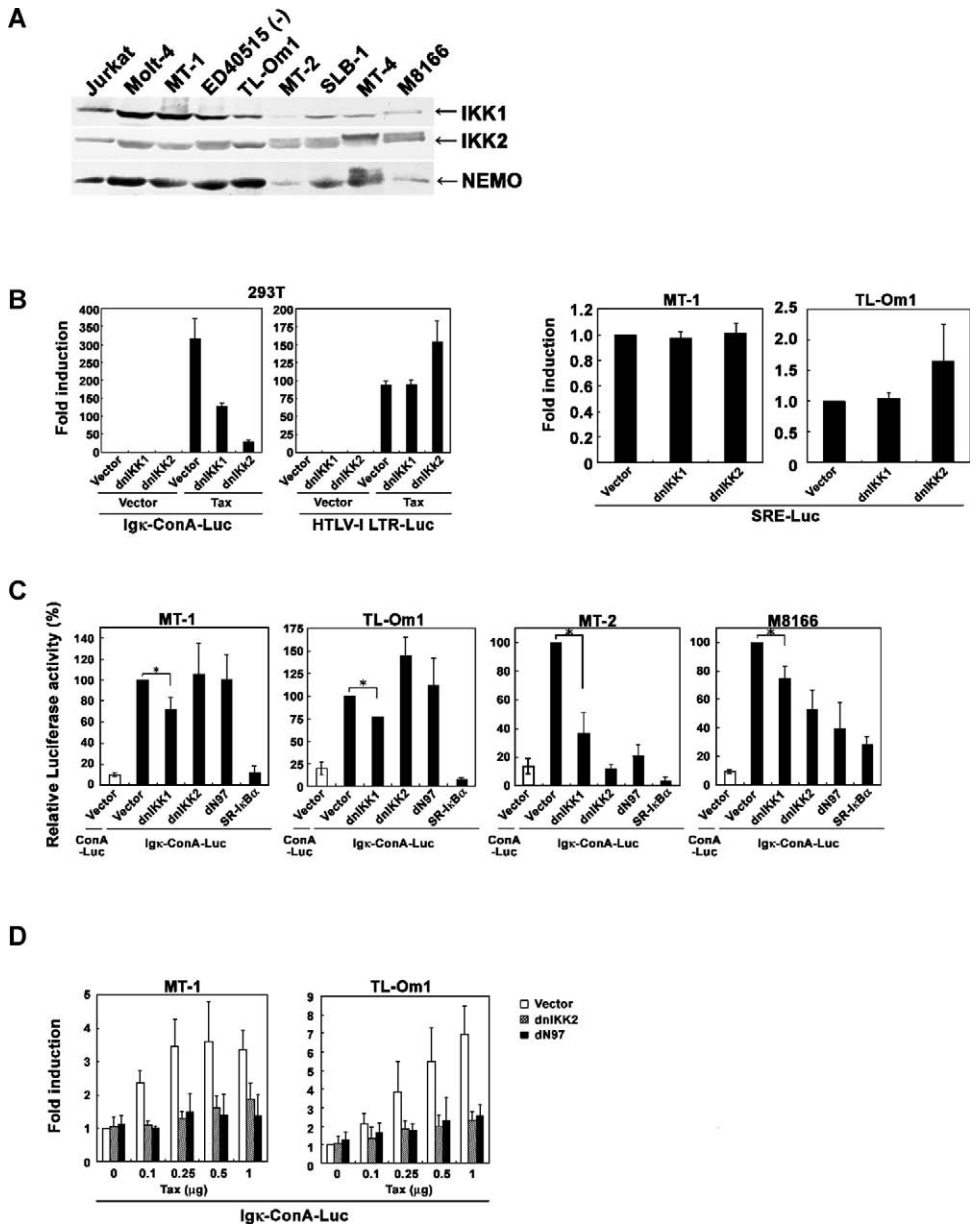

Figure 2. *IKK1, but not IKK2 or NEMO, is critically involved in NF-κB activation in ATL cells. (A) Steady-state levels of IKK1, IKK2, and NEMO in equivalent numbers of cells were determined by Western blotting using specific antibodies. (B) Specific inhibition of NF-κB–dependent transcription by dominant-negative forms of IKK. Left two panels: Approximately 2 × 10^5 293T cells were cotransfected with 0.1 μg of Igκ-ConA-Luc or HTLV-I LTR-Luc, 0.1 μg of EF1-LacZ, 1 μg of pcDNA3GVSV (vector), pcDNA3GVSV-IKK1DN (dnIKK1) or pcDNA3GVSV-IKK2DN (dnIKK2), and 0.1 μg of pCMV-Neo-Bam or pCMV-Neo-Bam-Tax. Right two panels: Approximately 5 × 10^6 MT-1 and TL-Om1 cells were cotransfected with 10 μg of pcDNA3GVSV, pcDNA3GVSV-IKK1DN, or pcDNA3GVSV-IKK2DN along with 5 μg of SRE-Luc and 3 μg of EF1-LacZ. Luc and β-gal activities were determined 40 hours after transfection for MT-1 and TLOm1 cells and 24 hours after transfection for 293T cells. Luc activity was normalized on the basis of β-gal activity. Each column represents the mean ± SD of three independent experiments. (C) Dominant-negative forms of IKK1 and IKK2 differentially suppress NF-κB activity in Tax-positive and Tax-negative HTLV-I–infected cells. Approximately 5 × 10^6 cells were cotransfected with 5 μg of ConA-Luc, 3 μg of EF1-LacZ, and 10 μg of pcDNA3GVSV (open column), or with 5 μg of Igκ-ConA-Luc, 3 μg of EF1-LacZ, and 10 μg of pcDNA3GVSV, pcDNA3GVSV-IKK1DN, pcDNA3GVSV-IKK2DN, pcDNA3-dN97 (dN97), or pRc-CMV-IκBαSR (IκBαSR) (filled columns). Relative Luc activity normalized to β-gal activity is presented as a percentage of the value obtained with Igκ-ConA-Luc and pcDNA3GVSV. Data are expressed as mean ± SD of three independent experiments. *P<.05 versus vector control. (D) Approximately 5 × 10^6 MT-1 and TL-Om1 cells were cotransfected with 5 μg of Igκ-ConA-Luc, 3 μg of EF1-LacZ, and 10 μg of pcDNA3GVSV, pcDNA3GVSV-IKK2DN, or pcDNA3-dN97 together with increasing amounts of pCMV-Neo-Bam-Tax. The total amount of DNA transfected was equalized with the pCMV-Neo-Bam vector. The panels show the relative Luc activity over the activity obtained with the combination of pcDNA3GVSV and pCMV-Neo-Bam vectors. Each column represents the mean ± SD of three independent experiments.*

TL-Om1 cells efficiently suppressed Tax-induced NF-κB–dependent transcription as expected, but did not affect the basal NF-κB activity in these cells, although we cannot completely rule out the trivial possibility that dN97 suppressed Tax expression in these cells (Figure 2*D*). Thus, dN97 was functionally expressed in MT-1 and TL-Om1 cells. These transfection studies strongly suggest that IKK1, but not IKK2 or NEMO, is important for NF-κB

activation in ATL cells, which contrasts with Tax-positive HTLV-I–transformed cells in which IKK1, IKK2, and NEMO play important roles for NF-κB activation.

Constitutive IKK Activity in ATL Cells

Recent studies have demonstrated two NF-κB activation pathways: one is NEMO-dependent, triggered by cytokines such as TNFα and IL-1β, and called the canonical pathway; the other is NEMO-independent and IKK1-dependent, triggered by B-cell activation factor (BAFF), lymphotoxinβ (LTβ), CD40, or TWEAK, and called the noncanonical pathway [69–73]. To further study the contribution of IKK1 to NF-κB activity in ATL cells, we determined IKK activity following immunoprecipitation by IKK1-specific antibody (Figure 3). Cell lysates prepared from equivalent numbers of cells were subjected to immunoprecipitation. One portion of the immunoprecipitates was used in *in vitro* phosphorylation assays with GST-IκBα or GST-IκBβ as substrates, and the remainder was subjected to immunoblotting for detection of immunoprecipitated IKK1. IKK1 was efficiently immunoprecipitated and the amounts of immunoprecipitated IKK1 were proportional to the expression levels of these molecules in each cell line (Figures 2*A* and 3). Thus, the band intensities of phosphorylated GST-IκBs are expected to represent IKK activity per cell. No phosphorylating activity on mutant GST-IκBα (S32A and S36A) or GST-IκB (S19A and S23A) was observed (data not shown). The results in Figure 3 indicated that IKK activity per cell was increased in both ATL cells and HTLV-I–transformed cells, compared with those in HTLV-I–free control T cells.

Aberrant Expression of NFKB2 p52 in ATL Cells

The recently identified noncanonical pathway of NF-κB activation involves the processing of NFKB2 p100 and the generation of p52. Although expression of p100 is controlled by NF-κB activity [74], the processing of p100 is a limited event that takes place independently of NEMO [36,37,40,73]. As this process is triggered by phosphorylation on the specific serine residues of p100 by IKK1, and not by IKK2 [39,40], we examined the generation of p52 in ATL cells (Figure 4). Consistent with a prior report that demonstrated Tax- and NEMO-dependent recruitment of IKK1 to p100, which resulted in the proteolytic generation of p52 [40], Western blot analysis of whole cell extracts prepared from equivalent numbers of cells detected p100 and p52 strongly expressed in Tax-positive HTLV-I–transformed cells. Although the control study (bottom panel) showed a lower amount of actin for Jurkat, which is smaller in size than the other cell lines, a longer exposure did not reveal significant p52 generation in this cell line. A polypeptide migrating slightly faster than p100 was recognized in MT-4 cells as reported previously [75], but its detailed character is not yet fully investigated. Aberrant expression of p100 and p52 was observed also in ATL cells, but not in HTLV-I–free Jurkat or Molt-4 cells. TL-Om1 cells appear to express polypeptides larger than p52, in addition to the full-length p100. Because the anti–p52 antibody used in this experiment was raised against the N-terminal 447 amino acids, they could be C-terminally truncated p100 products that are processed in a signal-independent manner, as was reported for Hut78 cells [35]. The aberrant p52 expression indicates that the noncanonical NF-κB activation pathway operates in Tax-negative ATL cells.

Specific Inhibition of NF-κB Activity Induces Cell Death in ATL Cells

Finally, we show that constitutive NF-κB activity is essential for survival or growth of ATL and HTLV-I–transformed T cells. This point has not been addressed by use of a specific NF-κB inhibitor IκBα, although previous reports suggested the importance of this transcription factor for survival of Tax-expressing T cells by use of drugs that suppressed NF-κB activity [27]. A SR-IκBα that cannot be phosphorylated at the specific serine residues targeted by IKK was expressed in HTLV-I–free Jurkat, ATL-derived MT-1, or HTLV-I–transformed MT-2 cells through retroviral gene transduction. Infection of Jurkat, MT-1, or MT-2 cells stably expressing the mEcoVR with retrovirus capable of expressing GFP alone, or SR-IκBα in combination with GFP, resulted in GFP expression in approximately 30% of cells. GFP-positive populations were sorted 72 hours after infection to selectively analyze infected cells. More than 80% of sorted cells were positive in GFP expression for each cell line. MT-1 and MT-2 cells expressing SR-IκBα were found to start dying 78 hours after infection (Figure 5*A*). In contrast, MT-1 and MT-2 cells expressing GFP alone remained viable and increased in number. However, Jurkat cells remained intact following SR-IκBα expression, and increased exponentially in number over the time studied (Figure 5*A* and data not shown). Because of rapid loss of MT-2 cell viability, preparation of MT-2 cell extracts and observation of MT-2 cells under UV microscope were done at an earlier time point than Jurkat and MT-1 cells. Figure 5*B* shows reduced NF-κB DNA-binding activity in SR-IκBα–transduced MT-1 and MT-2 cells, which correlated well with the increased steady-state levels of IκBα. Although NF-κB DNA-binding activity appears to be reduced by SR-IκBα expression in MT-2 cells to a lesser degree than in MT-1 cells, the viability of MT-2 cells was more rapidly and profoundly lost by SR-IκBα expression than that of MT-1 cells. The weak DNA-binding activity might remain in MT-2 cells partly because cells were harvested earlier than MT-1 cells by 16 hours. It is possible that MT-2 cells survive and proliferate, fully relying on Tax-induced NF-κB activity, and hence are more sensitive to NF-κB inhibition than Tax-negative MT-1 cells. However, MT-1 cells may have accumulated a variety of genetic and epigenetic changes supporting their survival and proliferation in the absence of Tax during the process of leukemogenesis, and could thereby be less sensitive to NF-κB inhibition. The nuclear morphology of the infected cells was examined by microscopic observation after staining with Hoechst33342 (Figure 5*C*). Nuclear condensation, a hallmark of apoptosis, was observed in MT-1 and MT-2 cells expressing SR-IκBα, but not in those expressing GFP alone or in Jurkat cells. These results clearly indicate that both ATL and HTLV-I–transformed T cells depend on elevated NF-κB activity for

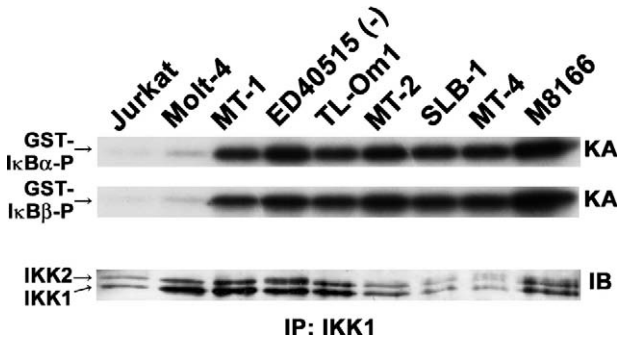

Figure 3. *Tax-independent IKK activation in ATL cells. Cytoplasmic extracts prepared from equivalent numbers of cells were immunoprecipitated with an IKK1-specific monoclonal antibody and subjected to in vitro kinase assay using GST-IκBα and GST-IκBβ as substrates, or to immunoblotting for detection of IKK1 and IKK2 in the precipitates with a mixture of IKK1- and IKK2-specific polyclonal antibodies. The experiments were carried out three times and the results were essentially reproducible.*

their survival, and thus implicate the importance of understanding the distinct mechanism of NF-κB activation in ATL cells.

Discussion

Several questions have remained to be solved about the NF-κB activity in ATL cell: Is NF-κB activation required for the development of ATL? How is NF-κB activated constitutively in the absence of Tax? Why is Tax expression often undetectable in ATL cells? We have demonstrated, through specific inhibition of NF-κB by SR-IκBα, that constitutive NF-κB activity is essential for the survival of ATL and HTLV-I–transformed cells (Figure 5). We have shown, for the first time, constitutive IKK activity in ATL cells that do not express detectable levels of HTLV-I gene products including Tax. Transfection studies suggest that this IKK activation is mechanistically different from that induced by Tax. We have also demonstrated aberrant expression of p52 in ATL cells in the absence of Tax, indicating that the recently described noncanonical NF-κB activation pathway operates in ATL cells. These results suggest that a Tax-independent cellular mechanism(s) rather than a tiny amount of Tax, if any, is responsible for the modification of IκB proteins and constitutive activation of NF-κB in ATL cells, which are eventually similar to those caused by Tax.

Constitutive NF-κB Activity Is Required for Survival of ATL Cells

We have extended our previous report that pharmacological inhibition of NF-κB induces apoptosis of HTLV-I–transformed cells and primary ATL cells [27] to formally demonstrate an essential role of NF-κB in their survival, by using SR-IκBα that specifically inhibits NF-κB. Pervious transfection studies revealed that IκBα was unable to inhibit transcriptional activity of p52/RelB dimer [76,77]. However, regulation of endogenous p52/RelB complexes is different from that of ectopically expressed p52/RelB in that inducible expression of p100 and RelB precedes the formation of p52/RelB complex. Based on our and other researchers' results, we envisage that phosphorylation of IκBα would be an important early step to induce the expression of p100 and RelB, which play central roles in the noncanonical pathway. Several cytokine signals (LTβ, CD40, and TWEAK) that activate this pathway also induce phosphorylation of IκBα and nuclear translocation of p50/RelA preceding the generation of p50/RelB and p52/RelB dimers [71–73,78–80]. In fact, we showed in Figure 1*C* phosphorylated IκBα in the cytoplasm of ATL-derived cells whose NF-κB activity is refractory to dominant-negative forms of IKK2 and NEMO, but sensitive to SR-IκBα (Figure 2*C*), suggesting that these cells have an IKK2- and NEMO-independent phosphorylating activity on IκBα. SR-IκBα is expected to block this step and shut off the NF-κB–induced production of p100 and RelB [74,81].

Finally, our supershift study revealed p50, p52, RelB, and a small amount of RelA in the NF-κB DNA-binding complexes of MT-1 cells (data not shown), which were all abolished by expression of SR-IκBα (Figure 5*B*). The p50/RelB dimer was previously shown to be sensitive to inhibition by IκBα in a transfection study [77]. SR-IκBα may inhibit p52/RelB and p50/RelB dimers at different steps and eventually ablate the entire NF-κB activity in MT-1 cells. Thus, our results suggest that loss of active NF-κB components including p52/RelB reduces the viability of ATL-derived cells. This promises well for the efficacy of an anti–NF-κB therapy against ATL; however, general inhibition of NF-κB by SR-IκBα, for instance, will not be appropriate for clinical use, unless SR-IκBα is targeted only to ATL cells. An NF-κB inhibitor specialized to ATL would be more useful. In this regard, Tax-mediated NF-κB activation attracted much attention, and efforts to elucidate its mechanism indeed led to the discovery of NEMO [53]. Nevertheless, because Tax expression is not detectable in primary leukemic cells or cell lines of leukemic cell origin, understanding the molecular mechanism of Tax-independent NF-κB activation in ATL cells rather than that of the Tax-dependent one should contribute more to establishing the molecular basis for the treatment of ATL.

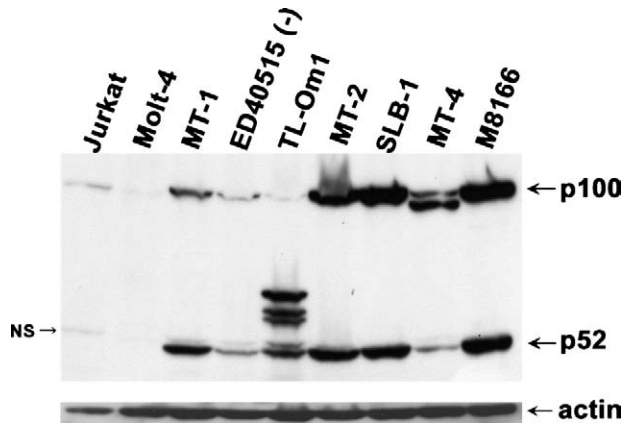

Figure 4. *Aberrant p52 expression in ATL cells. Whole cell extracts prepared from equivalent numbers of the indicated cells were analyzed by Western blotting using anti–p52 monoclonal antibody (C-5), and the same membrane was reprobed with an anti–actin antibody. Nonspecific bands are indicated as NS.*

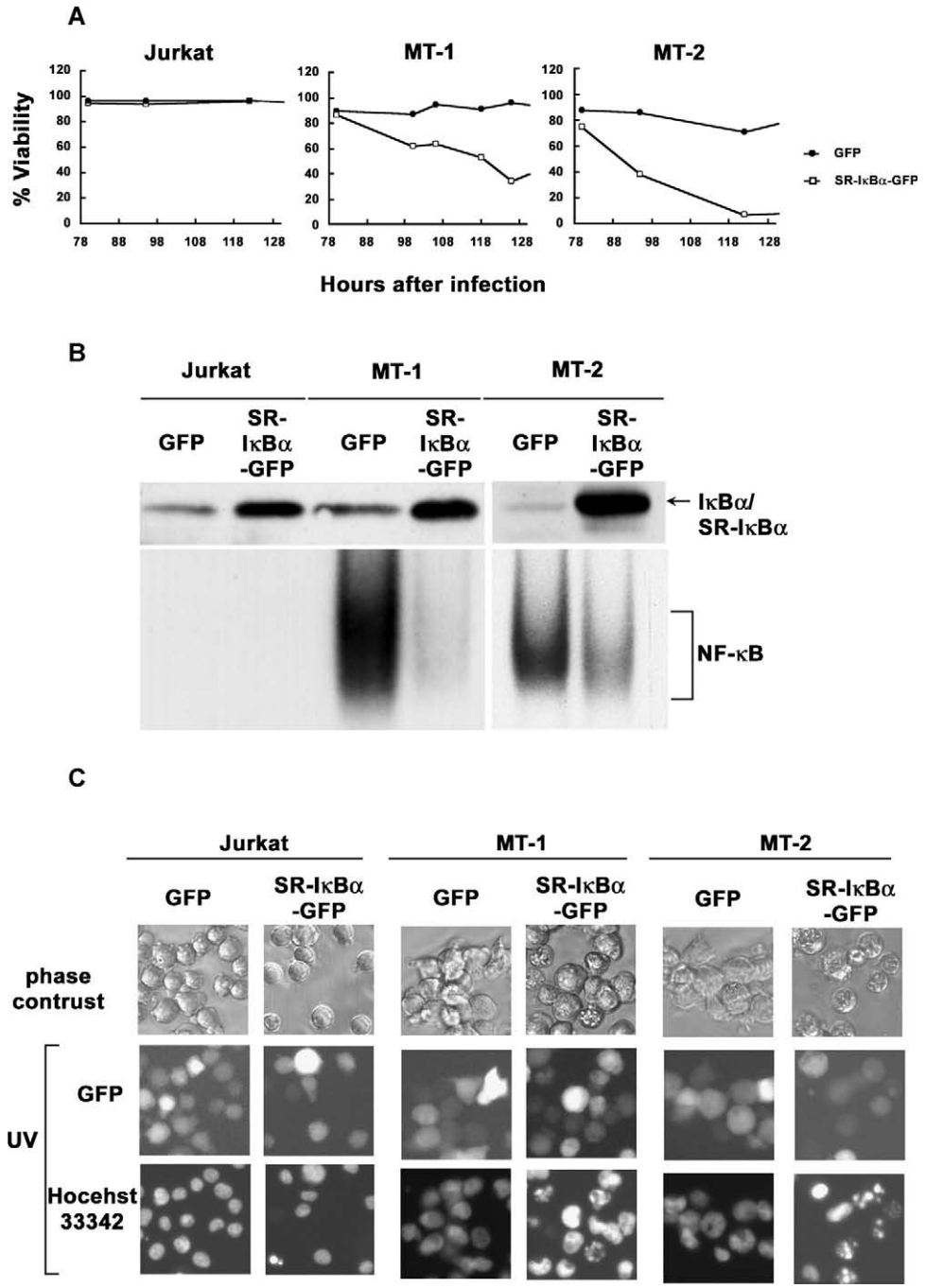

Figure 5. *Specific inhibition of NF-κB activity induces cell death in ATL cells. (A) Jurkat, MT-1, and MT-2 cells stably expressing mEcoVR were infected with retroviruses capable of expressing control GFP protein alone (GFP) or SR-IκBα together with GFP (SR-IκBα-GFP). GFP-positive populations were sorted and stained with trypan blue to assess cell viability. The results shown are representative of three independent experiments. (B) Cytoplasmic and nuclear extracts were prepared from equivalent numbers of sorted GFP-positive cells (Jurkat and MT-1: 2 × 10^5 cells, MT-2: 10^5 cells). Steady-state levels of IκBα in the cytoplasm were determined at 80 hours postinfection for MT-2 and 96 hours postinfection for Jurkat and MT-1 cells by immunoblotting using IκBα-specific antibody (upper panels). NF-κB DNA-binding activity was assessed by EMSA (lower panels). MT-2 cells were harvested at an earlier time point because of their rapid cell death. (C) Sorted cells were stained with Hoechst 33342 at 84 hours postinfection for MT-2 cell and at 108 hours postinfection for Jurkat and MT-1 cells, and photographed under three different conditions (phase contrast, UV–GFP, and UV–Hoechst 33342) with a confocal UV microscope. The experiments were carried out three times and the results were essentially reproducible.*

Tax-Independent IKK Activation in ATL Cells

We have presented functional evidence of distinct IKK activation in Tax-positive and ATL cells. Transfection studies in Tax-positive HTLV-I–transformed cells revealed that dnIKK1, dnIKK2, and dN97 NEMO efficiently suppressed NF-κB–dependent transcription, suggesting important roles of these molecules in Tax-mediated NF-κB activation. In contrast, in Tax-negative ATL cells, dnIKK2 and dN97 NEMO were unable to suppress NF-κB–dependent transcription, whereas dnIKK1 partially inhibited it (Figure 2*C*).

We also tested if simultaneous expression of dnIKK1 and dnIKK2 can suppress NF-κB activity in MT-1 and TL-Om1 cells. Transient transfection with 5 μg each of dnIKK1 and dnIKK2 constructs did not significantly reduce the reporter gene activity compared to that with 5 μg of vector and dnIKK1 constructs or that with 10 μg of dnIKK1 construct (data not shown). These observations suggest an important role for IKK1, but cannot exclude a possibility that NF-κB activity in these cells is partly IKK-independent. The role of IKK1 in NF-κB activation in ATL cells is further supported by the *in vitro* kinase assay results. IKK activities pulled down from ATL cells with anti–IKK1 antibody are comparable to those from Tax-positive HTLV-I–transformed cells except for M8166 cells. Moreover, consistent with a prior report showing that NF-κB activation by Tax involved phosphorylation of IKK2 and NEMO, we detected IKK2 phosphorylation, but not IKK1 phosphorylation, in Tax-positive HTLV-I–transformed cells, whereas IKK2 phosphorylation was not detected in ATL cells (data not shown). Collectively, these observations strongly suggest distinct IKK activation in ATL cells that are Tax- and NEMO-independent.

Despite the observed difference in IKK activation, IκBα and p100 were found modified similarly regardless of Tax expression, albeit to lesser extents in the absence of Tax. These observations raise a possibility that the functions of Tax in the IKK complex are, at least in part, achieved by a cellular mechanism in Tax-negative ATL cells. Xiao et al. [40] reported previously Tax-dependent recruitment of IKK1 to p100 that required NEMO and resulted in the generation of p52 following phosphorylation and subsequent processing of p100. Given the poor cotranslational processing of p100 [36,37], enhanced activation of IKK1-associated kinase activity, and generation of p52 in ATL cells, it is reasonable to postulate that the activated IKK1 in ATL cells phosphorylates p100 and triggers its processing in a Tax-independent manner. The features of IKK activation and modification of IκB proteins in ATL cells are most compatible to the recently described noncanonical pathway of NF-κB activation induced by BAFF, LTβ, CD40, or TWEAK that involves the phosphorylation-dependent generation of p52 in an IKK1-dependent, but NEMO-independent, manner [69–73,82].

Loss of the Viral Gene Expression During ATL Leukemogenesis

Based on the observation that Tax-independent constitutive NF-κB activity in cells of leukemic origin is essential for their survival, we hypothesize that these cells acquired this phenotype under the host's immunological surveillance. Tax is known to be a major target of cytotoxic T lymphocyte (CTL) immunity [21,83]; however, this CTL activity is poorly detectable in ATL patients [84–86]. In contrast, CTL activity against Tax is relatively high in HAM patients where HTLV-I–infected T cells express substantial levels of Tax. Tax is supposed to promote the proliferation of HTLV-I–infected T cells by altering the host gene expression in the early stages of leukemogenesis and also to enhance cellular gene mutations either by suppressing the expression of a DNA repair enzyme β-polymerase or by targeting key regulators of G1/S and M progression such as p16/INK4a, cyclin D1, D2, cyclin D3-cdk, and the mitotic spindle checkpoint apparatus [13–15,87–89]. However, expression of Tax should also activate CTL, which in turn would target Tax-positive cells for killing. This process may promote selective outgrowth of cells that acquired Tax-independent growth advantages such as constitutive NF-κB activity through alterations of host gene expression. This hypothesis can explain the long latency until the onset of diseases, relatively low incidence of developing ATL, monoclonal or oligoclonal growth of leukemic cells, and poor Tax-specific CTL activity in ATL patients. The constitutive NF-κB activity in a $CD4^+$ T-cell malignancy, cutaneous T-cell lymphoma (CTCL), would provide another piece of evidence that supports this hypothesis. CTCL has clinical and histopathological features quite similar to those of ATL. In fact, the only finding helpful for distinguishing ATL from CTCL is HTLV-I infection. A CTCL-derived cell line, Hut78, was reported to exhibit constitutive NF-κB activity that is essential for its survival, deregulated processing of p100, and aberrant expression of p52 [38,75,90]. Moreover, a rearranged NFKB2 gene product found in a CTCL patient that lacks portions of the ankiryn domain and IKK1 phosphorylation site localized in the nucleus [91]. Thus, the constitutive NF-κB activity associated with aberrant p52 expression may represent a common basis of these mature $CD4^+$ T-cell malignancies.

Noncanonical NF-κB Activation and Tumorigenesis

The relatively weaker expression of p100 in ATL cells compared to that seen in Tax-positive HTLV-I–transformed cells could be ascribed to the absence of strong activation of the canonical pathway, which is known to enhance p100 and RelB expression [74,81]. Nonetheless, the elevated expression of p52 in ATL cells implicates that the processing of p100 is an important event in the process of leukemogenesis. Interestingly, mice lacking the C-terminal half of p100, while still expressing p52, develop gastric and lymphoid hyperplasia, but not cancer [92]. In human lymphoid malignancies, a variety of rearrangement or deletion in the *nfkb2* locus can be found. Mutations cluster within the 3′ ankyrin-encoding domain of the *nfkb2* gene and lead to production of abnormal proteins, which results in constitutive p52 production and DNA-binding activity [34,35]. Deregulated production of p52 has also been reported in breast cancer cells and CTCL-derived cell lines [38,75,93]. These cancer cells may require an activity that induces the processing of p100 and disrupts its potent IκB function to keep the high NF-κB activity. The processing of p100 is often accompanied by nuclear translocation of RelB, whose expression is upregulated by NF-κB because p100, and not IκBα, specifically retains RelB in the cytoplasm [76]. Thus, not only the disruption of p100's IκB function, but also modifications of transcription by p52-containing or RelB-containing dimers could contribute to oncogenesis. However, these events do not appear to be sufficient for cancer development because none of the isolated p100 mutants or p52 can immediately induce cancer. The multistep development of ATL is therefore likely to involve yet unidentified molecular

events besides the constitutive NF-κB activation, but elucidation of the mechanism of the Tax-independent IKK activation in ATL cells will facilitate the understanding of the pathogenesis of ATL and should provide a rationale for establishing treatment of this incurable leukemia.

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