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Characterization of mRNA Expression of IκBα and NF-κB Subfamilies in Primary Adult T-cell Leukemia Cells

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Tax protein of HTLV-1 activates the transcriptional capacity of the NF-κB family, resulting in up-regulation of various genes, which are linked to phenotypic alterations of HTLV-1-infected T cells. To understand NF-κB regulation in HTLV-1-infected leukemic cells *in vivo*, we analyzed expression of NF-κB and IκBα in primary cells isolated from ATL patients. Using competitive polymerase chain reaction, we observed an elevated expression of IκBα mRNA in all four ATL cases tested. In contrast to the elevated mRNA levels, the levels of IκBα protein were remarkably reduced in some of these cases, suggesting destabilization of IκBα protein. On the other hand, mRNA expression of p50/p105 and p65, subfamilies of NF-κB, was enhanced in primary cells isolated from some ATL patients. Furthermore, the expression patterns of NF-κB subfamily were variable among patients and also different from those in T cells isolated from uninfected individuals. Although the number of cases analyzed was limited, we can conclude from these observations that activation of NF-κB is restricted to a few subfamilies *in vivo*. These findings *in vivo* are strikingly different from those in HTLV-1-infected T cell lines *in vitro*, in which Tax is responsible for NF-κB activation. It is therefore suggested that the elevation of NF-κB expression in leukemic cells of ATL patients might not be supported mainly by the viral protein Tax.

Key words: NF-κB — IκB — HTLV-1 — ATL

Adult T cell leukemia (ATL) is a malignancy of CD4-positive T cells[1] infected with human T cell leukemia virus type 1(HTLV-1).[2–4] The leukemic cells *in vivo* frequently express α chain of interleukin-2 receptor (IL-2Rα) at abnormally high levels.[5–7] Production of various lymphokines is also characteristic of ATL cells. These lymphokines include interleukin-1 (IL-1),[8–10] IL-2,[11] IL-6,[12, 13] tumor necrosis factor beta (TNFβ),[14] granulocyte-macrophage colony stimulating factor,[15] tumor growth factor,[16] ATL-derived factor,[17] and parathyroid hormone-related protein.[18] The expression of these genes has been shown to be partly regulated by the transcription factor family NF-κB.

Transcription factor NF-κB is negatively regulated by an inhibitory protein, IκBα, in resting T cells. Upon stimulation of T cells, IκBα protein is destabilized,[19–21] leading to the activation of NF-κB in response to the stimulus. The activated NF-κB also enhances expression of IκBα[20] to keep NF-κB activation transient. Tax protein, a transcriptional regulator of HTLV-1, induces destabilization of IκBα,[22, 23] and thus results in constitutive activation of NF-κB in HTLV-1-infected T cell lines, which leads characteristic changes of gene expression.[23]

Besides NF-κB, Tax also activates other transcription factors, CREB (cyclic AMP-response element binding protein)[24, 25] and serum response factor,[25, 26] which are involved in the activation of genes required for cell proliferation and viral replication. Based on these observations, Tax has been suggested to play critical roles in transformation of T cells and fibroblasts *in vitro*, tumor induction in Tax-carrying transgenic mice, and thus in induction of ATL.

However, the *HTLV-1* genes are not expressed significantly in primary leukemic cells isolated from ATL patients; the levels of expression are extremely low, and can be detected only by reverse transcriptase-mediated polymerase chain reaction (RT-PCR).[27] Since the viral expression *in vivo* is so different from those observed in cell lines *in vitro*, it is of interest to understand the differences of gene regulation between leukemic cells *in vivo* and cell lines established *in vitro*. We have analyzed the expression of NF-κB and IκBα in primary leukemic cells isolated from ATL patients and compared it with that in HTLV-1-infected T cell lines.

We report here that expression of IκBα mRNA, evaluated by RT-PCR, is elevated in leukemic cells of ATL patients so far tested. Furthermore, protein levels of IκBα were drastically reduced in some ATL cases. On the other hand, elevated expression of p50/p105 and p65 mRNA, subfamilies of NF-κB, was observed in primary cells of some cases. Furthermore, the expression patterns of NF-κB subfamily were different from those in uninfected T cells

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and from those observed in HTLV-1-infected T cell lines. These observations suggest that the regulation of NF-κB in leukemic cells in peripheral blood may not be mainly controlled by the viral protein Tax.

MATERIALS AND METHODS

Cells and RNA extraction MT-1, MT-2 and Hut102 are human T-cell lines infected with HTLV-1. Jurkat and CEM are human T-cell lines. These T-cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum.

Mononuclear cells of peripheral blood (PBMC) were obtained from venous blood by Ficoll-Hypaque gradient centrifugation. The final pellets of PBMC were directly frozen and kept at −80°C until use. The status of ATL and the white blood cell count for each sample were as follows: ATL-1, chronic ATL, 12,700/mm^3 (ATL cells: 74%); ATL-2, acute ATL, 142,000/mm^3 (ATL cells: 91%); ATL-3, acute ATL, 16,000/mm^3 (ATL cells: 55%); ATL-4, acute ATL, 228,000/mm^3 (ATL cells: 92%). For isolation of CD4-positive cells, PBMC were prepared from uninfected healthy individuals and immediately mixed with magnetic beads (Dynabeads M-450 CD4) that were coated with anti-CD4 antibodies. After processing according to the manufacturer's protocol, the cells bound to the beads were directly used for RNA preparation.

Total RNA was extracted from cells according to the AGPC method (acid guanidinium thiocyanate-phenol-chloroform method) described by Chomczynski and Sacchi.[28]

Primers for reverse transcription and competitive PCR Primers for the reverse transcription to prepare cDNA were random oligo-nucleotides, 5′-pd$(N)_6$ (Pharmacia Ltd., Tokyo). Primers for the competitive PCR to amplify the DNA sequence of 210–290 bp are summarized in Table I. The competitor DNAs were prepared by PCR according to Celi *et al.*[29] Briefly, the competitor DNA was shorter by 20–40 bases than the DNA sequence to be amplified by PCR and had the same primer binding sequences as the native template cDNA. To prepare such competitor DNA, we synthesized oligonucleotides with two specific sequences, one corresponding to the internal region of the amplified DNA and the other corresponding to either of the original primers in Table I. Using this hybrid primer and a partner of the original primers, PCR was carried out using the cDNA as a template to produce a shorter competitor DNA, which had the same primer binding sequences as for the following PCR.

Competitive RT-PCR Total cellular RNA equivalent to 5 $\times 10^4$ cells was converted into cDNA using reverse transcriptase ("Super Script" RNaseH-Reverse Transcriptase, Gibco BRL, Tokyo) according to manufacturer's protocol. Briefly, RNA and random primers were mixed and heated at 65°C for 10 min and then incubated with reverse transcriptase at 37°C for 1 h. cDNA was isolated by standard phenol extraction and used for the following PCR as a template. PCR was carried out by using slight modifications of the methods of Diviacco *et al.*[30] and Siebert and Larrick.[31] The cDNA was mixed with increasing doses of the competitor DNA and subjected to 35 cycles of PCR using reaction buffer containing 50 m*M* KCl, 10 m*M* Tris (pH 8.3), 1.5 m*M* $MgCl_2$, 0.25 m*M* deoxynucleoside triphosphate, 2.0 units of Taq polymerase, 1 mCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham, Tokyo), and 1 m*M* primers. Each PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 55°C for 75 s, and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. The amplified DNA products were separated by electrophoresis in 8% polyacrylamide gel and the radioactivity in each product corresponding to the native cDNA and competitor was estimated with an Image-Analyzer BAS2000 (Fuji Film, Tokyo).

Table I. Primer Sequences Used for the Competitive PCR

Target mRNA	Orientation	Primer sequence	Length of DNA (bp)	
			Amplified	Competitor
β-actin	S[a]	5′AAGAGAGGCATCCTCACCCT3′	218	188
	AS[a]	5′TSCSTGGCTGGGGTGTTGAA3′		
p52	S	5′CCATTGTGGAACCCAAGGAG3′	220	180
	AS	5′ACCAGGTCCACCTCGGGAAG3′		
p50	S	5′AAATGGTGGAGTCTGGGAAG3′	262	221
	AS	5′ACCGCCGAAACTATCCGAAAA3′		
c-Rel	S	5′TTGTGGAAGTGTCAGAGGAG3′	285	265
	AS	5′GGGTTCTGTGATAGCTTTGC3′		
p65	S	5′GACTACGACCTGAATGCTGT3′	223	190
	AS	5′CAATGTCCTCTTTCTGCACC3′		
IκBα	S	5′ACGAGCAGATGGTCAAGGAG3′	218	190
	AS	5′TGGAGTCTGCTGCAGGTTGT3′		

a) S and AS represent sense and anti-sense oligonucleotides, respectively.

Western blot analysis PBMC isolated from ATL patients and uninfected individuals were lysed by suspending the cells in RIPA buffer (0.01 *M* Tris-HCl, pH 8.0, 0.14 *M* NaCl, 0.5 % Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate). Proteins equivalent to 30 μg was subjected to immuno-blot analysis as described previously[22]. Rabbit polyclonal antibody against IκBα, C-21 (Santa Cruz Biotech., CA), was used as the first antibody.

RESULTS

Competitive RT-PCR of mRNA Numbers of primary cells isolated from ATL patients are generally limited, and therefore we used the competitive PCR method[30, 31] to estimate the expression of NF-κB and IκBα mRNAs. Total cellular RNA was prepared from mononuclear cells isolated from peripheral blood (PBMC) and used as a template for the reverse transcriptase. From the cDNA preparation, a specific cDNA sequence was amplified with a pair of primers that encompass an intron, so that the product could be distinguished from those of genomic DNA. PCR was carried out in the presence of increasing doses of a competitor DNA that has a sequence similar to, but shorter than that to be amplified by the PCR and can be amplified with the same primer set (Table I). The PCR products were analyzed by gel electrophoresis and the cDNA levels were calculated from the ratio between the two bands corresponding to those from native cDNA and competitor DNA (Fig. 1, A and B). To correct for the amount of RNA used for the assay and a possible effect of inhibitors on PCR, the expression levels of mRNA were calculated relative to that of β-actin mRNA.

Reproducibility and accuracy of the competitive RT-PCR were confirmed with every sample by adding increasing amounts of a competitor DNA to a constant amount of the native cDNA. Formation of the competitor product linearly increased with increasing dose of the competitor and there was a concomitant decrease of the product from native cDNA. Typical results for p65, a subfamily of NF-κB, are shown in Fig. 1B. From the point at which the amounts of products from the competitor and native cDNA are the same, the original amount of cDNA was determined. mRNA levels of IL-2Rα and p65 obtained by this competitive PCR were very similar to those obtained by the standard northern blot analysis (data not shown). These results revealed that our competitive PCR is reproducible and sensitive enough to quantify specific mRNA levels using 10^4 cells.

IκBα mRNA in primary ATL cells Mononuclear cells (PBMC) were isolated from peripheral blood of four ATL patients, ATL-1, -2, -3 and -4, and RNA was prepared immediately after the isolation of cells. The RNA samples were subjected to competitive PCR assay in duplicate to estimate the level of IκBα mRNA (Fig. 2A). The levels of IκBα mRNA were expressed relative to that of β-actin mRNA in the same sample, which was also estimated by competitive PCR. Since leukemic cells of ATL are CD4-positive T cells, the results were compared with those for CD4-positive T cells isolated from uninfected healthy individuals.

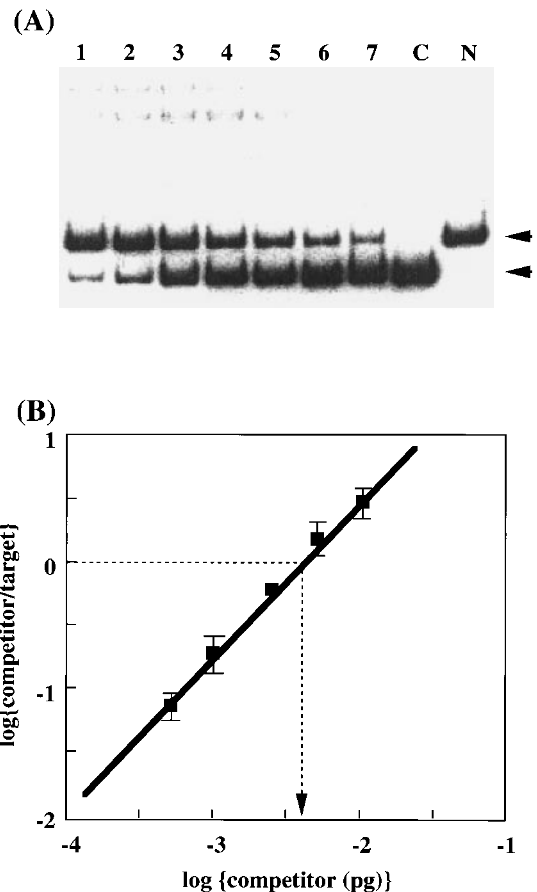

Fig. 1. A, Analysis of the products of the competitive RT-PCR of p65 mRNA. Lanes 1–7: A constant amount of cDNA preparation was mixed with increasing amounts of the competitor DNA. N and C represent DNA amplified from the normal p65 mRNA and competitor DNA, respectively. B, Dose dependency of the PCR products with increasing amounts of the competitor DNA. Each band in Fig. 1A was estimated by using an Image-Analyzer (BAS 2000) and the ratios of the amplified competitor DNA to the amplified target DNA were plotted against the amount of the competitor DNA used for the assay. The amount of specific cDNA in the sample was estimated as indicated by dotted lines based on the fact that equal amounts of the competitor DNA and cDNA gave the same amount of PCR products.

As can be seen in Fig. 2A, the levels of IκBα mRNA in primary cells from four ATL cases were significantly higher than those of CD4-positive T cells from healthy in-

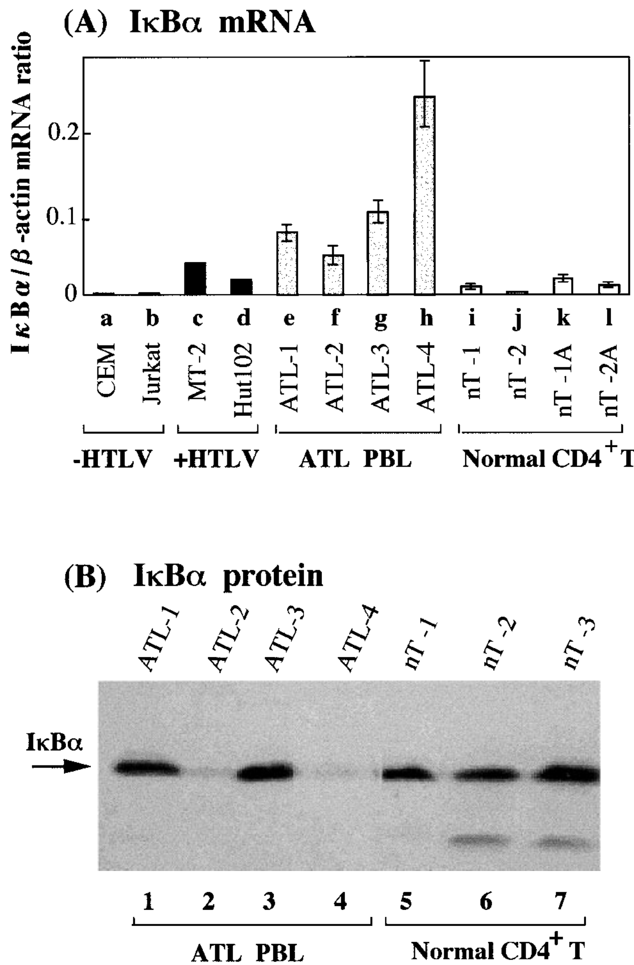

Fig. 2. Expression of IκBα at mRNA (A) and protein (B) levels in primary cells from ATL patients and normal individuals and in HTLV-1-infected T cell lines. A, mRNA levels of IκBα. The mRNA levels were estimated by competitive RT-PCR and expressed as the ratio to that of β-actin in the same sample. The assay was carried out in duplicate and the variations are indicated with horizontal bars. Uninfected T cell lines (a and b), HTLV-1-infected T cell lines (c and d), primary cells from ATL patients (e–h) and normal CD4-positive T cells from two normal individuals before (i and j) or after stimulation with PHA for 2 days (k and l) are indicated. B, Protein levels of IκBα. Total cell extract (30 μg protein) of the same samples as those in (A) was analyzed by western blotting using anti-IκBα antibodies. Lanes 1–4: Primary cells from ATL patients, ATL-1, -2, -3 and -4; lanes 5–7: unstimulated CD4-positive T cells from uninfected individuals. All samples were in the same gel, but the order of the lanes has been modified in the figure.

dividuals. The expression levels of IκBα mRNA in CD4-positive T cells were almost the same as those in whole PBMC of healthy individuals. Since primary cells from two cases, ATL-2 and -4, comprised 91 and 92% leukemic T cells with CD4-positive phenotypes, the elevated expression of IκBα mRNA observed here should reflect expression in the leukemic cells, but not other cells in the sample. In the other two cases, ATL-1 and -3, populations of leukemic cells were lower than in the former two cases, but they were still significantly high at 73% and 55%, so the high expression of IκBα mRNA should reflect increases in leukemic cells in the samples. These results are similar to the high expression of IκBα mRNA observed in HTLV-1-infected T cell lines (Fig. 2).[23]

It is known that expression of IκBα is enhanced upon stimulation of normal T cells to keep NF-κB regulation transient.[20] Leukemic cells of ATL were reported to have the phenotypes of activated or partially activated CD4-positive T cells. Because of this, IκBα expression in primary cells from ATL patients was compared with that of normal T cells treated with phytohemagglutinin (Fig. 2). The expression of IκBα mRNA increased upon stimulation, but the magnitudes of the effects were much less than the deviation in leukemic cells. Therefore, the results clearly demonstrated that the expression of IκBα mRNA in leukemic cells *in vivo* is abnormally high compared with those in resting and activated normal T cells.

It is worth noting here that the levels of IκBα mRNA in patients' PBMC were significantly higher than in HTLV-1-infected T cell lines, MT-2 and Hut102. In these cell lines, Tax protein destabilizes inactive complexes of IκBα/NF-κB and consequently activates NF-κB, which finally stimulates the expression of IκBα mRNA.[23] Therefore, the elevation of IκBα mRNA expression in patients' cells is extremely abnormal.

IκBα protein in primary ATL cells We previously demonstrated that Tax reduced protein levels of IκBα even when the mRNA expression was elevated, thus establishing constitutive activation of NF-κB in HTLV-1-infected T cell lines.[23] Therefore, protein levels of IκBα in primary cells from ATL patients were estimated by western blot analysis using the same samples as analyzed in Fig. 2A. As shown in Fig. 2B, whole cell lysates from two cases, ATL-2 and -4, gave a very faint band of IκBα protein (lanes 2 and 4). The intensities were much less than those of CD4-positive T cells and of the total PBMC of healthy individuals (lanes 5–7). The contents of leukemic cells in these two samples were 91 and 92%, so the low levels of IκBα protein should represent those in leukemic cells. In these primary cells, highly elevated mRNA expression was observed, so the low levels of the protein suggest the destabilization of IκBα protein. Destabilization of IκBα protein was similarly observed in HTLV-1-infected T cell lines, as previously described.[23] The other two cases, ATL-1 and -3 that contained 74 and 55% leukemic cells, however, did not show apparent reduction of the bands (Fig. 2B). The results may imply that IκBα protein was not destabilized in these

leukemic cells, but it is possible that the results were influenced by a lower content of leukemic cells. In support of this idea, it is noteworthy that the IκBα protein levels did not increase in parallel with the highly elevated expression of the mRNA (see Fig. 2A), which may suggest protein destabilization.

A faint but significant band which migrates much faster than IκBα protein was detected in some cases of normal CD4-positive T cells (lanes 6 and 7) , but not in samples with ATL (lanes 1–4). The band was also detected in a fraction of CD4-negative cells (data not shown). However, we have no information as to its origin, structure or significance in ATL.

Expression of NF-κB mRNA in ATL cells Higher levels of IκBα mRNA in all four cases with ATL suggested higher expression of NF-κB, which enhances transcription of the IκBα gene. To estimate NF-κB expression in primary cells, we used the same samples as used for the expression of IκBα, and carried out competitive PCR for mRNA of each of p50, p52, p65 and c-Rel (Fig 3). When the results were compared with those in CD4-positive T cells isolated from uninfected individuals, elevated expression of mRNA of the NF-κB subfamily was detected in some cases with ATL. The expression patterns of NF-κB subfamilies, however, varied from one patient to another (Fig. 3). Namely, p65 mRNA was highly expressed in ATL-3 and -4, and p50/p105 mRNA expression was moderately elevated in ATL-2 and -3 when compared with those in unstimulated CD4-positive T cells from uninfected individuals. In contrast to these observations, expression of c-Rel mRNA was rather suppressed in ATL-2, -3 and -4. Thus, no common pattern of NF-κB expression was observed among patients.

The NF-κB species highly expressed *in vivo* were strikingly different from those observed in infected T cell lines *in vitro*; p52 was expressed at extremely high levels in both cell lines, MT2 and Hut102, but it was not expressed at a high level in any ATL case so far tested. On the other hand, p65 was highly expressed in two cases with ATL, though it was not highly expressed in cell lines. Therefore, regulation of NF-κB expression in primary cells is rather different from that in infected cell lines.

DISCUSSION

Previously, we showed that HTLV-1 Tax disrupts feedback regulation of NF-κB through destabilization of IκBα,[23] thus establishing constitutive activation of NF-κB. However, primary leukemic cells isolated from ATL patients do not express Tax at a significant level.[27] Therefore, it is not clear whether the constitutive expression of NF-κB-directed genes *in vivo* can be explained by the action of Tax. In this study, we observed augmentation of IκBα expression at the mRNA level in primary leukemic cells. The

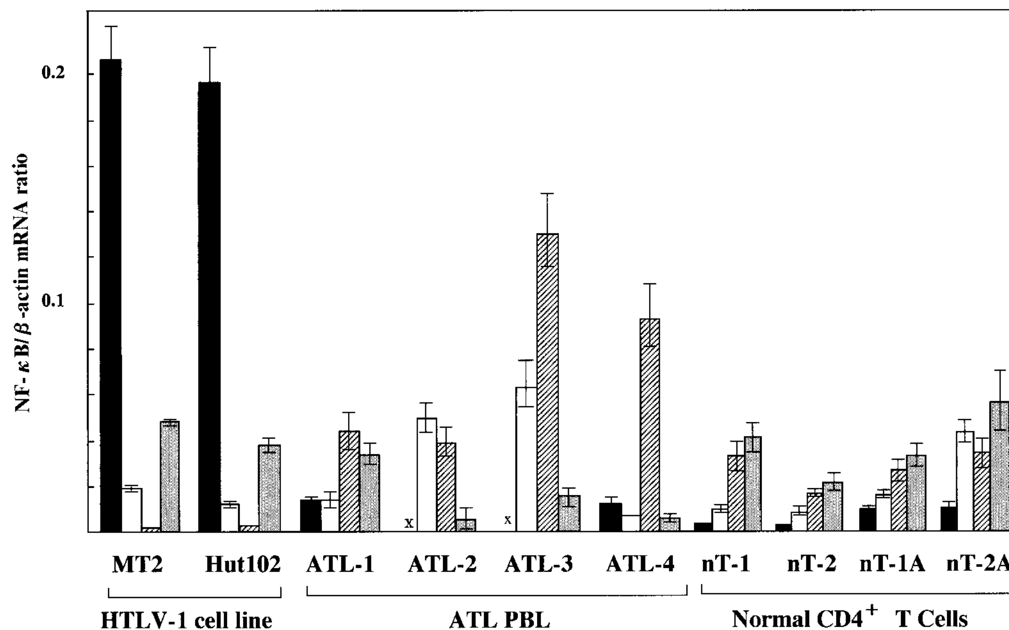

Fig. 3. Expression of NF-κB mRNA in primary cells from ATL patients, normal individuals and HTLV-1-infected T cell lines. The mRNA level of each subspecies of NF-κB, p50, p52, p65 and c-Rel, was estimated by competitive RT-PCR and indicated in the same way as in Fig. 2. Samples were the same as those in Fig. 2. ■, p52/p100; □, p50/p105; ▨, p65; ▩, c-Rel; x, undetectable.

levels of IκBα mRNA in primary leukemic cells were significantly higher than in activated T cells isolated from healthy individuals, so it was concluded that the expression of IκBα mRNA in leukemic cells is much higher than the normal levels in resting and activated T cells. These results were similar to those observed in HTLV-1-infected T cell lines.

In spite of the elevated levels of IκBα mRNA, reduction of IκBα protein was observed in some cases that harbored very high populations of leukemic cells (91 and 92%). These results clearly suggest destabilization of IκBα protein in primary cells. However, other two cases with 73 and 55% leukemic cells did not show apparent reduction of IκBα protein. Thus, destabilization of IκBα protein might not be general among ATL patients. However, since the protein levels were not elevated in parallel with the highly increased expression of the mRNA, the results may imply destabilization of IκBα protein in some extent. Although the number of cases analyzed here might be too small to allow any general conclusion, it appears that IκBα protein is destabilized in primary leukemic cells of at least some ATL cases.

Expression of IκBα mRNA is enhanced by activation of NF-κB, so elevated IκBα mRNA implies activation of NF-κB in primary leukemic cells. However, activation of NF-κB family in primary cells was detected only in some ATL patients. Furthermore, the elevation of NF-κB expression was restricted to p65 and p50/p105. These patterns of NF-κB expression are rather different from those in the HTLV-1-infected T cell lines; in two Tax-expressing T cell lines, MT-2 and Hut102, the patterns of NF-κB expression were very similar to each other, that is, p52/p100 expression is extraordinarily elevated, but p65 expression is suppressed (Fig. 3). However, in some cases of primary leukemic cells, p65 was highly expressed, but p52/p100 was not (Fig. 3). These results suggest Tax might not be a major factor regulating NF-κB expression in leukemic cells of ATL patients. In accordance with this conclusion, Tax expression in the primary cells isolated from these patients was extremely low and hard to estimate quantitatively with the assay used (data not shown). Thus, the activation pattern can not be extrapolated from those observed in HTLV-1-infected T cell lines. This further supports the idea that Tax might not be a major factor in the maintenance of the constitutive expression of NF-κB in leukemic cells in peripheral blood, although it might still be functional in other tissues such as lymphnode and skin.

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