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Constitutive Phosphorylation and Turnover of IκBα in Human T-Cell Leukemia Virus Type I-Infected and Tax-Expressing T Cells

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Human T-cell leukemia virus type I (HTLV-I) encodes a strong transcriptional activator, Tax, that stimulates transcription indirectly through the viral long terminal repeat and also activates a number of cellular genes via association with host transcription factors. The NF-κB/Rel pathway is a target for Tax *trans*-activation, and Tax has been correlated with increased NF-κB-binding activity and NF-κB-dependent gene expression in HTLV-I-infected cells. In this study we demonstrate that constitutive phosphorylation and increased turnover of the regulatory IκBα protein in HTLV-I-infected MT-2 and C8166 cells and Tax-expressing 19D cells contribute to constitutive NF-κB-binding activity, which consists primarily of c-Rel, p52(NFKB2), and p50(NFKB1). IκBα mRNA expression is also increased 7- to 20-fold in these cells, although the steady-state level of IκBα protein is reduced in HTLV-I-infected and Tax-expressing T cells. These results indicate that the viral Tax protein, by indirectly mediating phosphorylation of IκB, may target IκBα for rapid degradation, thus leading to constitutive NF-κB activity.

Transformation of T cells by the human T-cell leukemia virus type I (HTLV-I) represents the critical first step in the development of adult T-cell leukemia. The oncogenic potential of HTLV-I resides in the 40-kDa viral Tax protein, which has been characterized as a strong *trans*-activator of viral gene transcription, acting via 21-bp repeats within the long terminal repeat. Tax also *trans*-activates a number of cellular genes which probably play a role in leukemogenesis. Since Tax is not a DNA-binding protein per se, Tax *trans*-activation occurs indirectly via preexisting host transcription factors (for reviews, see references 9 and 32). Several reports have demonstrated a physical interaction between Tax and host proteins that are targets for *trans*-activation, including $p67^{SRF}$, cyclic AMP-responsive element-binding protein, Ets-1, TATA-binding protein, activating transcription factor, and NF-κB/Rel proteins (6, 8, 10, 13, 17, 18, 22, 35, 36, 39).

The NF-κB/Rel transcription factors are a family of dimer-forming proteins that bind to the consensus DNA sequence 5′-GGGANNYYCC-3′. NF-κB/Rel proteins are pleiotropic transcriptional regulators of cellular and viral genes implicated in immunoregulatory and inflammatory processes (reviewed in references 14 and 25). Structurally, all DNA-binding members of the family (p50 [NFKB1], p52 [NFKB2], p65, c-Rel, and RelB) share an amino-terminal *rel* homology domain involved in DNA binding, protein dimerization, and nuclear translocation (reviewed in references 14 and 25). In unstimulated cells, NF-κB activity is regulated, in part, by posttranslational mechanisms. NF-κB/Rel proteins exist in the cytoplasm coupled to inhibitory molecules, collectively termed IκB, that are responsible for cytoplasmic retention of NF-κB. IκB also constitutes a family of proteins including IκBα, IκBγ, bcl-3, p105, and p100, characterized by the presence of five to seven repeats of a 33-amino-acid ankyrin motif. IκBα specifically binds to and masks the nuclear localization signal of NF-κB/Rel proteins, thereby preventing nuclear translocation (reviewed in references 4 and 12). After cellular stimulation by multiple inducers (cytokines, virus infection, phorbol esters, mitogens), activation of signal transduction cascades leads to the phosphorylation and subsequent degradation of IκBα (5, 7). Release of IκBα permits NF-κB/Rel dimer translocation to the nucleus and target gene activation (reviewed in references 4 and 12). Degradation and resynthesis of IκBα appear to be general mechanisms determining the rapid but transient activation of gene activity by NF-κB (7, 31, 34). NF-κB/Rel activity is also regulated at the transcriptional level, conferred by the presence of NF-κB sites in the promoters of NFKB1 (p105 and p50), c-*rel*, MAD-3 (IκBα), and NFKB2 genes (15, 23, 26, 34, 37).

Previous studies demonstrated that Tax-expressing and HTLV-I-infected T cells exhibited constitutive NF-κB-binding activity, composed of c-Rel, p52(NFKB2), and p50(NFKB1) (3, 20, 22, 24, 40). A Tax-dependent correlation was established between expression of NFKB2(p100 and p52), induction of c-Rel, and *trans*-activation of NF-κB-mediated gene expression. Furthermore, NFKB2(p100) physically associated with c-Rel and with Tax in HTLV-I-infected cells (6, 22). To further understand Tax–NF-κB/Rel interactions, we have examined regulation of IκBα activity in HTLV-I-infected T cells (MT-2, MT-4, and C8166-45), Tax-expressing Jurkat cells (19D), and Jurkat cells.

Constitutive NF-κB-binding activity in HTLV-I-infected cells. In unstimulated Jurkat cells (Fig. 1A, lane 1), no NF-κB-specific protein-DNA complexes were detected in nuclear extracts by mobility shift analysis (1, 6, 20, 22, 40) with a ^{32}P-labeled probe containing the human immunodeficiency virus type 1 enhancer (5′-AGGGACTTTCCGCTGGGGACT TTCC-3′). After phorbol myristate acetate (PMA) treatment for 2 h, NF-κB DNA-binding activity was induced at least 20-fold (lane 2). Shifted-shift analysis with NF-κB-specific antibodies (29) demonstrated that p65, p50, and c-Rel were the main components of the protein-DNA complex (Fig. 1A, lanes

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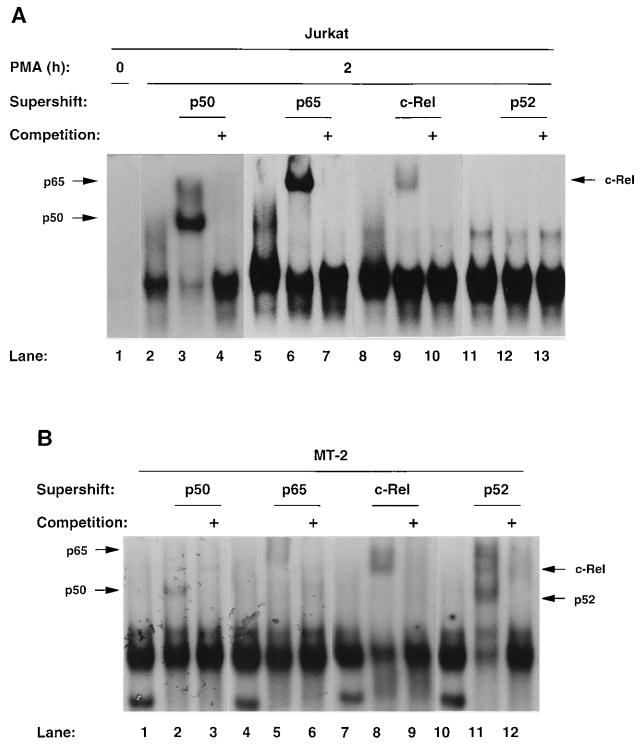

FIG. 1. NF-κB-binding activity in Jurkat and MT-2 cells. Nuclear extracts were prepared from untreated or PMA-treated cells (final PMA concentration, 25 ng/ml) as previously described (28). Nuclear extracts (5 μg) were incubated with a ^{32}P-radiolabeled HIV-1 enhancer probe and analyzed by electrophoretic mobility shift assay (21). In shifted-shift reactions, antibody specific for each NF-κB subunit (indicated above the lanes) was incubated with extract prior to probe addition. For peptide competition (indicated as + above the lanes), both antibody and peptide (1 μg) were incubated with extracts before probe addition (22). Arrows indicate the antibody-protein-DNA complex. (A) Jurkat cells. Extracts from untreated (lane 1) or 2-h PMA-induced (lanes 2 to 13) Jurkat cells were used. Lanes 1 to 4 represent a 16-h exposure; lanes 5 to 13 represent a 60-h exposure. (B) MT-2 cells. Extracts from uninduced MT-2 cells were used.

3, 6, and 9); no reactivity with p52 antibody was detected (Fig. 1A, lane 12). The specificity of the shift reactions was verified by competition with an excess of peptide against which the antiserum was raised (Fig. 1A, lanes 4, 7, 10, and 13). In contrast, constitutive NF-κB-binding activity in MT-2 cells was composed predominantly of c-Rel, p52, and p50; with MT-2 extracts, these antibodies partially inhibited formation of the NF-κB–DNA complex and also produced a shifted complex (Fig. 1B, lanes 2, 5, 8, and 11). As demonstrated above, the relative amount of p65-binding activity was decreased in MT-2 cells compared with Jurkat cells (compare Fig. 1A, lane 6, and Fig. 1B, lane 5), suggesting that either p65-binding activity or protein expression was altered in HTLV-I-infected T cells.

Constitutive IκBα phosphorylation. Since phosphorylation and degradation of IκBα have been implicated as critical steps leading to NF-κB activation following induction (5, 7, 31, 34), we examined the possibility that the constitutive NF-κB-binding activity observed in HTLV-I-infected and Tax-expressing cells correlated with the modification of IκBα phosphorylation state and increased IκBα turnover. Extracts from untreated or PMA-treated Jurkat and MT-2 cells were examined for the presence of phosphorylated forms of IκBα by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting with an IκBα-specific antiserum, essentially as described previously (29). Phosphorylated IκBα was not detected in Jurkat cells following PMA treatment for times ranging from 0 to 60 min (Fig. 2A, lanes 1 to 7; a dark exposure is shown). On the other hand, a band migrating just above IκBα was detected immediately after PMA addition to MT-2 cells (Fig. 2A, lane 10). Most importantly, darker exposure of the immunoblot revealed the slower-migrating band in untreated MT-2 cells, indicating a constitutive level of IκBα phosphorylation in MT-2 cells (Fig. 2A, lane 8). PMA treatment of MT-2 cells increased IκBα levels and also increased the amount of the putative phosphorylated form (Fig. 2A, lanes 10 to 15). To ensure that the slower-migrating form represented phosphorylated IκBα, MT-2 extracts (0 and 30 min) were treated with calf alkaline intestinal phosphatase (CIP) or CIP plus phosphatase inhibitors to dephosphorylate IκBα in vitro (Fig. 2B). Dephosphorylation was achieved by incubating extracts (100 μg) with 5 U of CIP for 1 h at 37°C. Another reaction mixture contained both CIP and phosphatase inhibitors (10 mM sodium vanadate and 50 mM sodium fluoride) to block in vitro dephosphorylation of IκBα, as previously described (5). The disappearance of the upper band with CIP treatment (Fig. 2B, lanes 2 and 5) and its reappearance in the presence of CIP plus inhibitors (Fig. 2B, lanes 3 and 6) demonstrated that the slower-migrating band is phosphorylated IκBα. Appropriately exposed autoradiograms were scanned by laser densitometry to determine the ratio of phosphorylated to unphosphorylated IκBα. The ratio of IκBα to phosphorylated IκBα varied from about 5:1 in unstimulated MT-2 cells (Fig. 2A, lanes 10 and 11) to 2:1 in MT-2 cells treated with PMA for 60 min (Fig. 2A, lane 15). These results indicated that phosphorylated IκBα was present constitutively in HTLV-I-infected cells.

To extend the observation that Tax expression correlated with the presence of constitutively phosphorylated IκBα, similar analyses were performed with Tax-expressing 19D Jurkat cells (Fig. 2C) and HTLV-I-infected cell lines C8166-45 and MT-4 (Fig. 2D). Unphosphorylated IκBα was detectable in 19D cells, and immediately after addition of PMA, induced expression of phosphorylated IκBα was also detectable (Fig. 2C, lanes 1 and 2); this result was in contrast to Jurkat cells, in which phosphorylated IκBα was not detected (Fig. 2A). PMA treatment further increased expression of phosphorylated IκBα in 19D cells without affecting the levels of the nonphosphorylated form (Fig. 2C, lanes 3 to 6). Laser densitometry established that the IκBα-to-phosphorylated IκBα ratio was 10:1 after induction with PMA (Fig. 2C).

C8166-45 cells, derived from human cord blood lymphocytes cocultured with adult T-cell leukemia-derived cells, carry a deleted HTLV-I genome and express Tax and Rex proteins only (30), whereas MT-4 cells, although transformed by HTLV-I, do not express Tax (data not shown). Phosphorylated IκBα was easily detected in C-8166-45 cells by immunoblot analysis (Fig. 2D, lane 3), as well as in MT-2 cells (Fig. 2D, lane 4). Control reactions with CIP and phosphatase inhibitors demonstrated the phosphorylated nature of the upper band (data not shown). Interestingly, MT-4 cells did not express any detectable phosphorylated IκBα (Fig. 2D, lane 5). Taken together, these results demonstrate that expression of Tax proteins in T cells is correlated with the presence of phosphorylated IκBα.

Figure 2D also illustrates that treatment of Jurkat cells with tumor necrosis factor alpha (140 U/ml) for 5 min resulted in the appearance of a slower-migrating phosphorylated IκBα species (Fig. 2D, lane 2). This phosphorylated IκBα form appeared to be quite unstable, since it was not detected after 15 min of tumor necrosis factor alpha treatment, as described previously (34), or after PMA treatment of Jurkat cells (Fig. 2A).

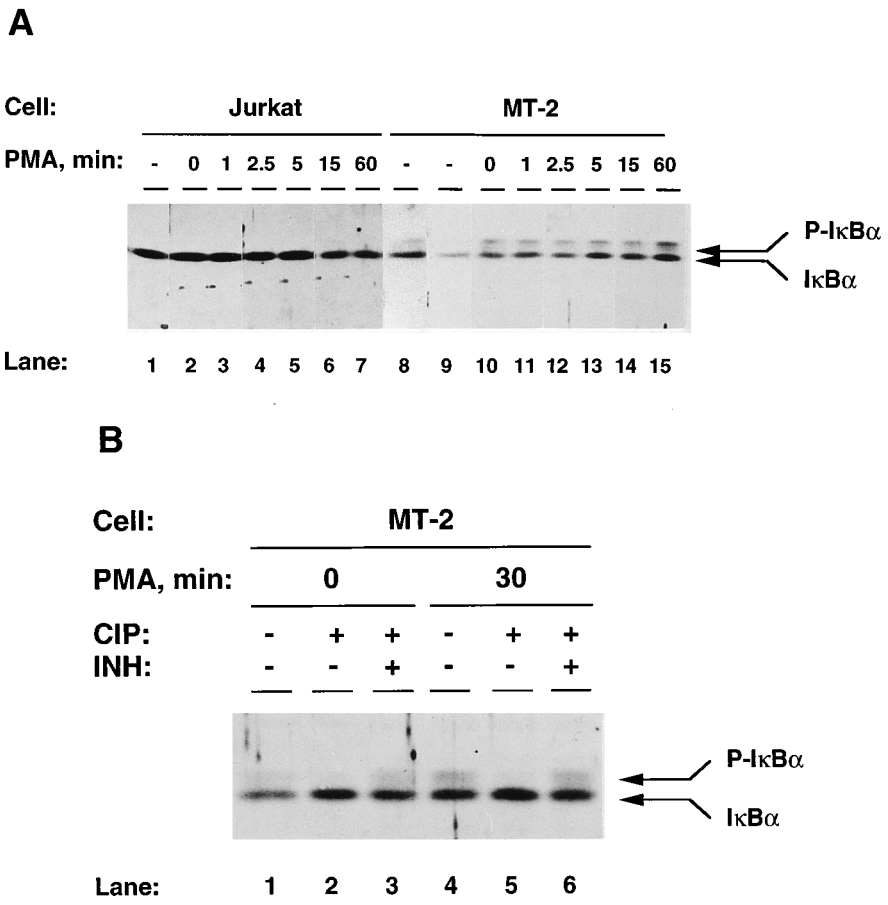

FIG. 2. Phosphorylated IκBα in HTLV-I-infected and Tax-expressing T cells. (A) Detection of phosphorylated forms of IκBα. Whole-cell extracts were prepared from untreated Jurkat (lane 1) and MT-2 (lanes 8 and 9) cells and from cells treated with PMA for 0 (lanes 2 and 10), 1 (lanes 3 and 11), 2.5 (lanes 4 and 12), 5 (lanes 5 and 13), 15 (lanes 6 and 14), and 60 (lanes 7 and 15) min. Denaturing electrophoresis was performed on 20-cm SDS–12% polyacrylamide gels. After transfer to nitrocellulose, IκBα signals were detected by immunoblotting and chemiluminescence (ECL; Amersham). The phosphorylated form of IκBα is indicated as P-IκBα. Lane 8 represents a darker exposure of lane 9. (B) In vitro dephosphorylation of phosphorylated IκBα. Extracts from MT-2 cells treated for 0 (lanes 1 to 3) and 30 (lanes 4 to 6) min with PMA were incubated at 37°C for 1 h in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5, and 6) of 5 U of CIP. Inhibitors of phosphatases (10 mM sodium vanadate and 50 mM sodium fluoride) were also included in reactions loaded on lanes 3 and 6. After incubation, SDS sample buffer was added and reactions were immediately loaded on SDS–12% polyacrylamide gels. IκBα signals were detected as described above. (C) Phosphorylated IκBα in Tax-expressing 19D cells. Whole-cell extracts were prepared from untreated 19D cells (lane 1) and from cells treated with PMA for 0, 5, 15, 30, and 60 min (lanes 2 to 6, respectively). Samples were examined for phosphorylated IκBα as described above. Lane 7 is a positive control loaded with MT-2 cell extracts. (D) Analysis of phosphorylated IκBα in untreated and tumor necrosis factor alpha (TNFα)-treated Jurkat cells and in untreated C8166-45, MT-2, and MT-4 cells. Extracts were prepared and analyzed as described previously (22). The amount of extract loaded in each well was normalized to obtain similar signal intensities: lane 1, untreated Jurkat, 100 μg; lane 2, 5 min TNFα-treated Jurkat cells, 100 μg; lane 3, untreated C8166-45 cells, 200 μg; lane 4, untreated MT-2 cells, 100 μg; lane 5, untreated MT-4 cells, 50 μg.

Increased IκBα turnover in HTLV-I-infected cells. We next examined whether increased phosphorylation in MT-2 and C8166-45 cells was accompanied by increased turnover of IκBα. IκBα protein expression and turnover were analyzed by immunoblotting in Jurkat and MT-2 cells treated with the protein synthesis inhibitor cycloheximide (50 μg/ml) for times ranging from 0 to 8 h (Fig. 3). Whole-cell extracts were prepared and analyzed for IκBα by immunoblotting as described in the legend to Fig. 3. IκBα signals were quantified by

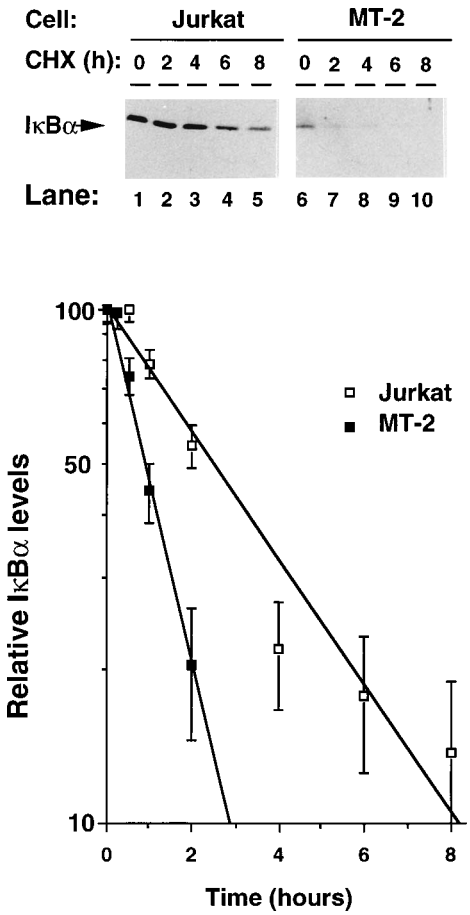

FIG. 3. IκBα turnover in Jurkat and MT-2 cells. Jurkat cells (lanes 1 to 5) and MT-2 cells (lanes 6 to 10) were treated with cycloheximide (50 μg/ml) for times ranging from 0 to 8 h, as indicated above the lanes. Whole-cell extracts (20 μg) were analyzed for IκBα by immunoblotting with an IκBα-specific antibody (AR20) and a chemiluminescence detection system (Amersham). Bands corresponding to the IκBα signal were quantified by laser densitometry and IκBα half-life was determined; the graph represents the average of three separate experiments. The level of IκBα in Jurkat (□) and MT-2 (■) cells at a given time, divided by the IκBα level at time zero, is plotted.

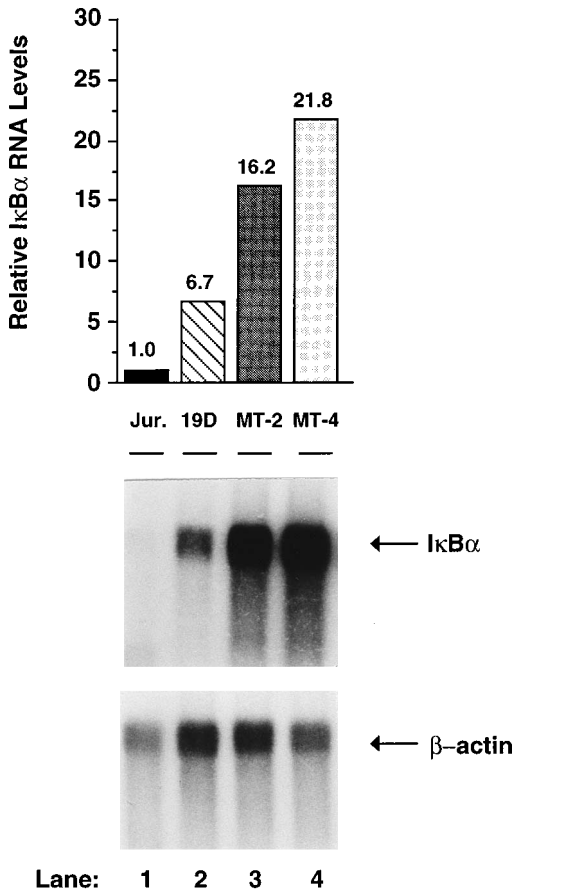

FIG. 4. Northern blot analysis of MAD-3 (IκBα) gene expression. Poly(A)$^+$ RNA (5 μg) was prepared from Jurkat (lane 1), 19D (lane 2), MT-2 (lane 3), and MT-4 (lane 4) cells as described previously (21). After resolution by formaldehyde agarose gel electrophoresis, RNA was transferred to nylon membrane and immobilized by UV light exposure. The filter was hybridized with a ^{32}P-labeled *Rsa*I fragment of MAD-3(IκBα) and with a β-actin probe as internal control. The signals were quantified by laser densitometry; IκBα levels were expressed relative to β-actin mRNA.

laser densitometry and plotted against time of treatment. Turnover rates were averaged from two or three separate experiments. Jurkat cell extracts contained sevenfold more IκBα protein than MT-2 cells did (Fig. 3, lanes 1 and 6). Also, C8166-45 cells contained about two- to threefold less IκBα protein than MT-2 cells (NP; data not shown). Furthermore, the turnover of IκBα was more rapid in MT-2 cells (Fig. 3, lanes 7 to 10) than in Jurkat cells (Fig. 3, lanes 2 to 5).

The graph shown in Fig. 3 represents a quantitative analysis of the decay of IκBα in Jurkat and MT-2 cells derived from three independent experiments. The half-life of IκBα in unstimulated Jurkat cells was calculated as 2.6 ± 0.1 h. In PMA-treated Jurkat, MT-2, and C8166-45 cells, the half-life of IκBα was reduced three- to fourfold to 1.0 ± 0.2 h, 0.85 ± 0.1 h, and 0.72 ± 0.3 h ($P < 0.01$), respectively. These results demonstrate that IκBα turnover is increased threefold in PMA-treated, HTLV-I infected, and Tax-expressing cells and support the model that phosphorylation of IκBα may target IκBα for rapid degradation (5, 7, 31, 34).

Overexpression of IκBα mRNA in HTLV-I-infected and Tax-expressing cells. One consequence of constitutive NF-κB-binding activity is increased expression of NF-κB-regulated genes (1, 20, 24, 27, 40), including transcriptional induction of the MAD-3(IκBα) gene itself (5, 7, 23, 31, 34). Therefore, to examine whether rapid turnover of IκBα and increased NF-κB-binding activity correlated with increased transcription, MAD-3(IκBα) expression was analyzed by Northern (RNA) blotting with poly(A)$^+$ mRNA (5 μg) from Jurkat, Tax-expressing 19D, and HTLV-I-infected (MT-2 and MT-4) T cells (Fig. 4). An *Rsa*I fragment of IκBα (16), labeled by random priming with [α-^{32}P]dCTP, served as IκBα-specific probe. RNA signals were scanned by laser densitometry, normalized to β-actin expression, and plotted as relative mRNA levels. MAD-3(IκBα) gene activity was strikingly higher in Tax-expressing and HTLV-I-infected cells than in Jurkat cells. IκBα mRNA levels were 7- to 20-fold higher in 19D, MT-2, and MT-4 cells (Fig. 4, lanes 2 to 4) than in normal Jurkat cells (lane 1). In similar experiments, the mRNA levels of NFKB1, *relA*, and c-*rel* genes were also transcriptionally stimulated about two to fourfold in MT-2 and MT-4 cells compared with Jurkat cells (data not shown). Thus, decreased IκBα protein level detected in MT-2 cells is not due to impaired MAD-3 gene activity; rather, IκBα mRNA expression is upregulated by constitutive NF-κB activity observed in Tax-expressing cells.

The present study demonstrates that constitutive phosphorylation and increased turnover of IκBα protein occur in HTLV-I-infected and Tax-expressing T cells. In general, an inverse correlation between Tax protein and steady-state levels of IκBα was observed in these studies; i.e., higher levels of Tax in HTLV-I-infected cells resulted in lower levels of IκB and higher levels of phosphorylated IκBα species. IκBα mRNA transcript levels were also increased 7- to 20-fold in Tax-expressing cells, probably as a consequence of constitutive NF-κB-binding activity and induction of the IκBα gene. These results support a model in which a HTLV-I Tax-mediated phosphorylation of IκBα targets IκBα for degradation, possibly by a ubiquitin-dependent proteolytic pathway (16). Disruption of the NF-κB/IκB autoregulatory pathway results in constitutive NF-κB DNA-binding activity that may promote aberrant NF-κB-dependent gene expression in T cells (1, 6, 20, 22, 40).

Despite increased IκBα mRNA expression in HTLV-I-infected MT-2 and MT-4 cells, IκBα protein levels were on average sevenfold lower than in Jurkat cells; this apparent discrepancy was partially resolved by the rapid turnover of IκBα in these cells. In unstimulated Jurkat cells, the half-life of IκBα was 2.6 h (156 min), while in MT-2 cells, the half-life of IκBα was reduced to 0.85 h (51 min). However, the activity of the pathway(s) involved in IκBα degradation must also be increased at least 10-fold in HTLV-I-infected cells to account for the rapid and continuous turnover of IκBα. A similar conclusion was also reached in a recent study of NF-κB activity and IκBα turnover during B-cell differentiation (27).

In HTLV-I-infected and Tax-expressing cells, alterations in NF-κB subunits involved in DNA binding have been reported. In Jurkat cells, p50 and p65 subunits represented the main DNA-binding components early after induction (Fig. 1); at later times, c-Rel and p50 were the abundant DNA-binding subunits, in part because of the transcriptional induction of c-Rel (22, 24). In Tax-expressing and HTLV-I-infected cells, c-Rel, p52(NFKB2) and p50(NFKB1) were the major DNA-binding components (22). Although p65 protein levels were similar or elevated in HTLV-I-infected cells compared with those in Jurkat cells (data not shown), p65 binding was reduced, indicating that p65 activity was sequestered in HTLV-I-infected cells. Several possibilities may account for decreased p65-binding activity: (i) p65 may be targeted for rapid degradation because of its association with phosphorylated IκBα; (ii) despite its rapid turnover, IκBα may be able to sequester p65 in the cytoplasm; (iii) competition for target sites by other NF-κB proteins such as c-Rel and NFKB2 may limit p65 activity; or (iv) as suggested by a recent study (34), p65 could be sequestered in HTLV-I-infected cells by the NFKB2 p100 precursor rather than by IκBα. This last suggestion is attractive, since p100 levels are increased in HTLV-I-infected cells and p100 is the main NF-κB subunit with which Tax associates in vivo (6, 22).

Interestingly, pre-B-to-B-cell differentiation involves changes in NF-κB activity that are closely analogous to those described in T cells versus HTLV-I-infected T cells. NF-κB activity is highly inducible in pre-B cells and in normal T cells but constitutive in both mature B cells and Tax-expressing T cells. In the last two cell types, MAD-3 and c-*rel* mRNAs are overexpressed and c-Rel is a major component of the constitutive DNA-binding activity. Importantly, IκBα turns over rapidly with a half-life between 40 and 50 min in mature B cells and Tax-expressing T cells, whereas in pre-B and normal T cells it is between 150 and 160 min. Moreover, in mature B cells, increased IκBα turnover is due to increased activity of a serine protease, since tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK), an inhibitor of serine proteases, stabilizes IκBα and inhibits NF-κB-binding activity (27). The recent observation that HTLV-I Tax interacts with the ankyrin repeat domain of the p105 precursor of IκBγ (17, 18) suggests that Tax-IκB interactions may influence the phosphorylation state of IκB. However, it should be noted that Tax-IκBα interactions have not been demonstrated (22, 29). An alternative model is that HTLV-I Tax may physically associate with and activate a host kinase that phosphorylates IκBα; phosphorylation may target IκBα for degradation by serine protease(s). One candidate kinase is the 68-kDa double-stranded RNA-dependent kinase (19) which binds to Tax in vitro (data not shown). Tax would thus disrupt IκB–NF-κB interactions and release free NF-κB/Rel and Tax complexes. Other activities associated with Tax, such as transcription factor dimerization (2, 39) and/or transcriptional activation (10, 11, 38), would then promote Tax-mediated gene activity.

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