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Kinetic Analysis of Human T-Cell Leukemia Virus Type I Tax-Mediated Activation of NF-κB

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The human T-cell leukemia virus type I (HTLV-I) Tax protein induces the expression of cellular genes, at least in part, by activating the endogenous NF-κB transcription factors. Induced expression of cellular genes is thought to be important for transformation of T cells to continued growth, a prelude to the establishment of adult T-cell leukemia. However, neither underlying mechanisms nor kinetics of the Tax-mediated activation of NF-κB are understood. We have analyzed a permanently transfected Jurkat T-cell line in which the expression of Tax is entirely dependent on addition of heavy metals. The initial NF-κB binding activity seen after induction of Tax is due almost exclusively to p50/p65 heterodimers. At later times, NF-κB complexes containing c-Rel and/or p52 accumulate. The early activation of p50/p65 complexes is a posttranslational event, since neither mRNA nor protein levels of NF-κB subunits had increased at that time. We demonstrate for the first time a Tax-induced proteolytic degradation of the NF-κB inhibitor, IκB-α, which may trigger the initial nuclear translocation of NF-κB. As nuclear NF-κB rapidly and potently stimulates resynthesis of IκB-α, the steady-state level of IκB-α does not significantly change. Thus, the dramatic Tax-induced increase in the IκB-α turnover may continually weaken inhibition and activate NF-κB. Additional, distinct actions of Tax may contribute further to the high levels of NF-κB activity seen.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent for adult T-cell leukemia (reviewed in reference 66). Along with standard retroviral genes coding for Gag, Pol, and Env, the pX region of the HTLV-I genome encodes several unique products, including the Tax protein. Tax is essential for viral gene expression, mediating its effects on transcription through cyclic AMP (cAMP)-responsive elements (CRE) in the viral long terminal repeat (LTR) (36). Several lines of evidence indicate that Tax is essential also for initiation of cellular transformation by HTLV-I (28, 54). Tax is known to induce many cellular growth-promoting genes, including genes encoding the T-cell growth factor interleukin-2 (IL-2) (34), the α subunit of the IL-2 receptor (4, 43, 57), and the c-*fos* proto-oncogene (22). Also, Tax can induce expression from the LTR of human immunodeficiency virus type 1 (HIV-1) in dually infected cells (10).

The activation of cellular and viral genes by Tax is mediated through at least three distinct *cis*-acting DNA sites: CRE (e.g., those present in the HTLV-I LTR and in the c-*fos* promoter), serum-responsive elements (e.g., that present in the c-*fos* promoter), and κB elements (e.g., those present in the IL-2 and IL-2 receptor α-subunit promoters and in the HIV-1 LTR). Distinct domains of Tax are important for activation through CRE and κB sites, as revealed by mutational analysis (64, 65); this finding suggests distinct mechanisms of action. While Tax does not bind to DNA directly on its own, it does harbor potential transactivation domains (24). Tax interacts with certain cellular transcription factors which bind the elements through which Tax function is mediated (8, 23, 69, 70, 74, 75); therefore, Tax may function as an accessory protein in the transactivation of select genes. In particular, Tax may do so together with the CRE-binding protein CREB, a constitutive nuclear protein with limited transactivation potential unless posttranslationally activated through a cAMP-responsive pathway (73). Tax has also been reported to exert its effects by enhancing the dimerization efficiency of many (if not all) DNA-binding proteins of the basic-leucine zipper class, including CREB (3, 71, 75).

NF-κB is a family of dimeric transcription factor complexes, composed by many combinations of members of the Rel/NF-κB family of polypeptides, including, in vertebrates, p50, p52 (p50B, p49, lyt-10), RelA (p65), c-Rel, and RelB (reviewed in references 13, 29, and 30). The predominant complex in most cells is p50/p65 (p50/RelA). It is retained in the cytoplasm along with other dimeric complexes by their inhibitor IκB-α. Various cell stimuli, including mitogens, cytokines, or physical stress, lead to proteolytic degradation of IκB-α and concomitant translocation of NF-κB complexes into the nucleus (7, 15, 16, 18, 33, 67). Once in the nucleus, these complexes transactivate through κB sites by virtue of transactivation domains found in p65 (RelA) (5, 61), c-Rel (35), or RelB (59). It has been suggested that, in addition to the pool of IκB-α inhibited complexes, there may exist other cytoplasmic pools of complexes, kept there by the p105 and p100 molecules, the precursors for p50 and p52, respectively (47, 52, 53, 56, 60, 68). These precursors contain IκB-α-like inhibitory domains in their C-terminal halves.

Although Tax has been known to activate NF-κB, neither the precise nature of the complexes activated nor the molecular mechanism by which Tax functions is understood. It has been suggested that Tax may function indirectly by inducing transcription of NF-κB components (2, 44). Other studies provide evidence for a posttranslational mechanism (45, 46), including the potential involvement of physiological pathways which activate NF-κB in response to extracellular signals (62). This latter conclusion was based on the partial inhibition of Tax-mediated activation of NF-κB by the ion chelator pyrrolidinedithiocarbamate (PDTC), although its precise mode of action in cells is not well understood. It has also been suggested

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that Tax acts by interacting with p50 subunits, bound to their cognate κB DNA sites (70). While the functional significance of this observation remains to be evaluated, Tax clearly is able to recruit NF-κB complexes into the nucleus (2, 4, 40, 43, 44) (see below). None of the previous studies have provided direct molecular explanations for this phenomenon. In addition, the kinetics of activation of NF-κB by Tax have not been described, an important point given the dynamic nature of NF-κB activity, which is subject to multiple feedback regulatory mechanisms (reviewed in references 13, 29, and 30).

In this study, we have analyzed the Tax-mediated activation of NF-κB with a permanently transfected Jurkat T-cell line in which expression of Tax protein is induced by heavy metals (50, 55). Initially p50/p65 was activated and recruited into the nucleus at least in part from cytoplasmic IκB-α-associated pools. We directly demonstrate a Tax-induced degradation of preformed IκB-α and a concomitant loss of preformed IκB-α associated with p65. Despite the increased IκB-α turnover, overall IκB-α levels are maintained as a result of the rapid induction of IκB-α mRNA and protein synthesis immediately following nuclear NF-κB translocation.

MATERIALS AND METHODS

Cell lines and transfections. JPX-9 and JPX/M cells (50, 55), Jurkat 19D cells (72), and Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics. JPX-9 and JPX/M cells were incubated with 120 μM $ZnCl_2$ to induce the functional Tax protein and a mutant Tax protein, respectively.

Nuclear and whole-cell extract preparation. Nuclear extracts were prepared as described by Abmayr et al. (1). Whole-cell extracts were prepared by repeated freeze-thawing in a buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.3 M KCl, 0.75 mM $MgCl_2$, 0.1 mM EDTA, 0.05 mM EGTA, 12.5% glycerol, and protease inhibitors (Boehringer protease inhibitor kit) followed by ultracentrifugation.

Electrophoretic mobility shift assay (EMSA). Nuclear and whole-cell extracts (4 μg) were incubated for 30 min in 10 μl of buffer containing 0.1 M KCl, 20 mM HEPES, 0.2 mM EDTA, 0.1 μg of poly(dI-dC) (Pharmacia) per ml, 1 μg of bovine serum albumin per ml, 0.5 mM dithiothreitol, and a ^{32}P-labeled palindromic κB probe (6, 11). Electrophoresis and supershifting analysis using 1 μl of antibodies were performed as previously described (11).

Northern (RNA) analysis. Twenty micrograms of total RNA, prepared as described previously (17), was fractionated on an agarose formaldehyde gel and blotted onto a nylon membrane. The membrane was sequentially hybridized with ^{32}P-labeled probes. The probes used were p50 (nucleotides 200 to 810 [14]), p52 (nucleotides 140 to 1198 [11]), p65 (631 nucleotides from the ATG start codon [58]), IκB-α (nucleotides 254 to 1550 [32]), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Clontech).

Western blot (immunoblot) analysis. Whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membranes by using a semidry blotter (Bio-Rad), and analyzed with the ECL Western blotting detection system (Amersham) as instructed by the manufacturer.

Pulse-chase experiments and immunoprecipitation. JPX-9 and JPX/M cells were starved for 15 min in methionine- and cysteine-depleted medium and pulse-labeled for 1 h with 200 μCi each of [^{35}S]methionine and [^{35}S]cysteine per ml. The cells were then washed, resuspended in complete medium, and divided into two groups: one group was treated with 120 μM $ZnCl_2$, and the other was untreated. The cells were harvested after 0, 6, 9, and 12 h, and whole-cell extracts were prepared. Coimmunoprecipitations using anti-p65 and anti-p50 antibodies were performed on these cell extracts in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA. The antigen-antibody complexes were precipitated with protein A-Sepharose beads and analyzed by SDS-PAGE and fluorography.

Antibodies. The antibodies used were an antipeptide antibody directed against the N-terminal 13 amino acids of p50 (12, 20, 21), a p52 monoclonal antibody which recognizes an epitope in the N terminus (12), an antipeptide antibody directed against the N-terminal 14 amino acids following the initiator methionine of p65 (21), a p65 rabbit polyclonal antibody (12, 20), a c-Rel antipeptide antibody (antibody 265 in reference 56; kindly provided by N. Rice), a rabbit polyclonal antibody directed against the C-terminal portion of IκB-α (15), and a Tax antipeptide antibody (kindly provided by J. N. Brady). The specificities of the antibodies were demonstrated in several ways, including their recognition of only the specifically targeted protein but not that of any other related Rel or IκB family member expressed in vitro or exogenously expressed in NTera-2 cells, which are otherwise devoid of NF-κB/IκB proteins (12, 20, 21). In the case of peptide antibodies, their specificities were also demonstrated by blocking with the original peptides.

RESULTS

Activation of NF-κB by Tax in JPX-9 Jurkat cells. JPX-9 Jurkat cells are permanently transfected with a metallothionein promoter-driven Tax expression vector (55). The expression of the Tax protein was induced with $ZnCl_2$ from undetectable levels to easily demonstrable levels within 6 h of treatment, increasing to maximal levels within 12 to 18 h (Fig. 1A, lanes 1 to 6). As a result of the expression of Tax, nuclear NF-κB activity was activated and easily demonstrated with an EMSA by 12 but not by 6 h of treatment with $ZnCl_2$ (Fig. 1A, lanes 7 to 12). No NF-κB binding activity could be detected in untreated cells, correlating with the absence of detectable Tax proteins in such cells. The kinetics of NF-κB activation indicate a delay in the response to Tax, which we estimate to be on the order of 2 to 3 h (data not shown; see below). This delay may have been due to a slow mode of action of Tax or to the requirement for a threshold level of Tax protein necessary for full function, a possibility since levels of Tax were still rising at the time nuclear NF-κB activity emerged.

Importantly, the observed strong activation of NF-κB was due to the Tax protein and not to the treatment with $ZnCl_2$ by itself. JPX/M, a Jurkat cell line permanently transfected with a nonfunctional Tax protein (50), did not show such activation in response to $ZnCl_2$ (Fig. 1A, lanes 13 to 18). Nonetheless the JPX/M cells were fully competent to activate levels of NF-κB equal to those seen in JPX-9 cells or parental Jurkat cells when other signals such as tumor necrosis factor alpha, phorbol myristate acetate (PMA), and/or phytohemagglutinin (PHA) were used (data not shown). It has been reported previously that heavy metals can be weak inducers of NF-κB binding activity in some cells (26). The mutant Tax-containing Jurkat cells used here did not significantly activate NF-κB after $ZnCl_2$ treatment alone. At times, barely detectable NF-κB activity was present within 1 or 2 h of treatment in both Jurkat lines with higher metal concentrations; however, such weak activation had dissipated entirely by 6 h. The strong Tax-mediated NF-κB activity was comparable in quantity to that seen with

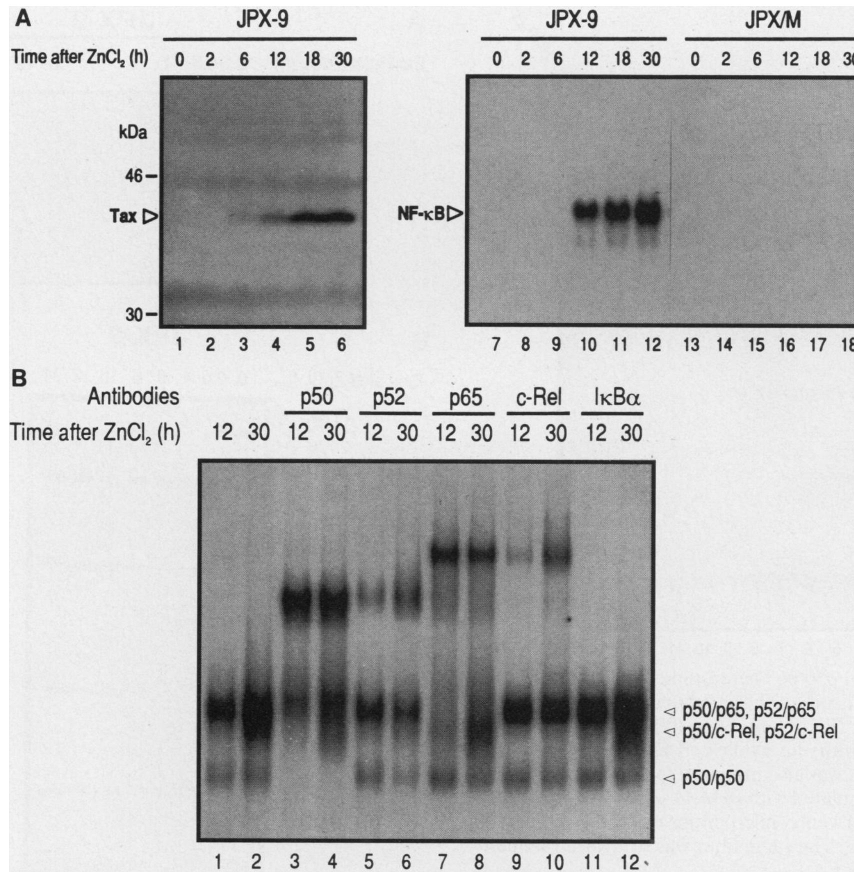

FIG. 1. Identification of the individual subunits of NF-κB activated by Tax. (A) JPX-9 and JPX/M cells were treated with $ZnCl_2$ for the periods indicated, and the whole-cell and nuclear extracts were prepared simultaneously. The whole-cell extracts from JPX-9 cells were separated by SDS-PAGE (10% gel) and analyzed by Western blotting with anti-Tax antibody (lanes 1 to 6). The nuclear extracts from JPX-9 and JPX/M cells were incubated with a ^{32}P-labeled κB palindromic probe and analyzed by EMSA (lanes 7 to 18). (B) The JPX-9 nuclear extracts obtained with 12 and 30 h of $ZnCl_2$ treatment were incubated with the indicated antibodies before addition of the ^{32}P-labeled κB palindromic probe and analyzed by EMSA (lanes 3 to 12). For lanes 1 and 2, no antibody was included in the reaction. Positions of complexes without antibodies are shown at the right.

maximal levels of tumor necrosis factor alpha or PHA plus PMA. While NF-κB binding activity increased up to 30 h of treatment, Tax expression had clearly plateaued by 18 h. Longer exposures of the EMSA results also revealed a faster-migrating band shift in addition to the much stronger NF-κB shift; that shift was due to p50 homodimers, as shown previously (20, 21) and as shown below.

The NF-κB complexes activated early by Tax consist primarily of p50/p65 heterodimers. NF-κB-like binding activities can be composed of any number of heterodimers and/or homodimers of proteins belonging to the Rel/NF-κB family. The binding complexes which were activated by Tax and thus translocated to the nucleus were analyzed for their subunit compositions in EMSAs with the use of subunit-specific supershifting antibodies (Fig. 1B). The palindromic probe used here is particularly well suited to detect the various NF-κB complexes (6, 11, 20, 25, 39). Extracts of JPX-9 cells, treated for 12 or 30 h with $ZnCl_2$, were used in EMSAs together with an antibody to p50, p52 (p50B), p65, or c-Rel. The major bandshift seen at 12 h was almost completely supershifted with p65 antibodies and largely supershifted with p50 antibodies. Since the p50 antibodies are known not to be completely effective in supershifting p50/p65 heterodimers (21), and since the other antibodies recognized only a minor portion of the 12-h complexes, we conclude that p50/p65 heterodimers were the initial primary target for activation by Tax. The minor complexes which contributed to the major bandshift at this time point appeared to be mostly p52/65 heterodimers. The weak faster-migrating shift was due to p50 homodimers, since only p50-specific antibodies could supershift it.

In contrast to the predominance of p50/p65 at early times after Tax induction, analysis at later times revealed significant quantities of other complexes which contained c-Rel and/or p52 (Fig. 1B). Because of the presence of multiple complexes, the 30-h major shift was more diffuse. The complexes migrating slightly faster than p50/p65 contained primarily heterodimers of c-Rel with p50 and/or p52, while the much less prominent slower complexes included most likely p65 homodimers, c-Rel homodimers, and c-Rel/p65 heterodimers, as judged by the supershift analysis and the known relative migrations of various complexes (25, 31, 51). The anti-RelB antibody did not generate any significant supershifts at any time, indicating that RelB did not significantly contribute to the NF-κB activity after Tax induction in these cells (data not shown). An anti-IκB-α control antibody also did not produce any supershift, as expected, confirming that IκB-α was not part of the activated complexes. Stimulation of Jurkat cells with PHA, by comparison, led to activation of p50/p65 heterodimers within

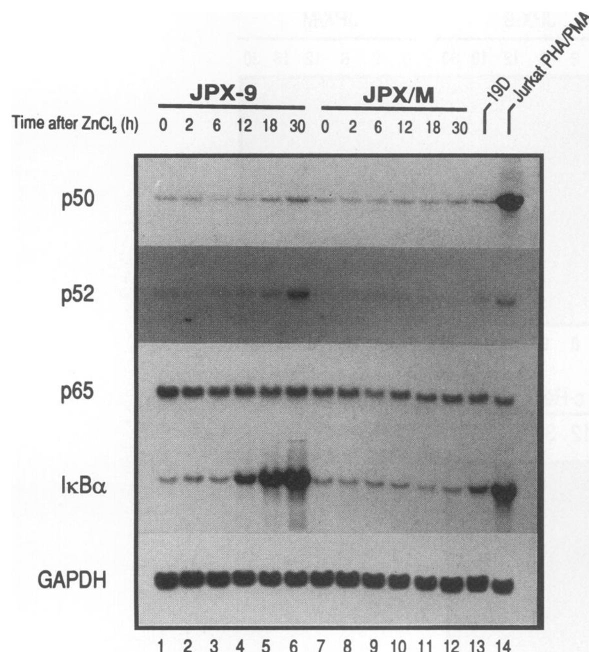

FIG. 2. Initial activation of p50/p65 heterodimers by Tax is not due to increased mRNA levels encoding these subunits. Total RNA was prepared from $ZnCl_2$-treated JPX-9 (lanes 1 to 6) and JPX/M (lanes 7 to 12) cells simultaneously with the whole-cell and nuclear extracts analyzed in Fig. 1. Total RNA was also prepared from Jurkat 19D cells (lane 13) and Jurkat cells stimulated with PHA (1 μg/ml) and PMA (20 ng/ml) for 4 h (lane 14). Twenty micrograms of total RNA was analyzed by Northern blotting. The same filter was hybridized sequentially with the indicated probes.

30 min, followed by the appearance of nuclear complexes containing c-Rel and p52 by about 8 h (data not shown).

While p50/p65 heterodimers were major contributors to the NF-κB binding activity at both 12 and 30 h after Tax induction, the c-Rel- and p52-containing complexes changed from minor to major contributors during this time frame. Potentially distinct mechanisms of Tax could have effected activation of the two types of complexes. In the studies described below, we concentrated on the early activation of p50/p65 to elucidate the mechanisms by which Tax might function.

Initial activation of NF-κB by Tax does not proceed via induced expression of the proteins which make up NF-κB activity. Previous reports suggested that Tax may activate NF-κB activity by selectively upregulating the expression of components for NF-κB (2, 44). Therefore, the mRNA levels for various subunits of NF-κB complexes were analyzed during a time course of Tax activation (Fig. 2). No changes in mRNA levels were observed until after NF-κB was activated. Thus, while p50/p65 activity was easily demonstrated in these same cells by 12 h (activity could be detected as early as 8 h; see below), the mRNA levels for p50 and p52 did not begin to change until about 18 h, while p65 levels remained steady throughout. Levels of c-Rel mRNA had increased by about 30 h (data not shown). IκB-α mRNA levels, however, had increased dramatically at about the time NF-κB was activated, reflecting most likely the direct transcriptional stimulation of this gene by NF-κB itself (15, 16, 18, 19, 42, 63, 67). For comparison, the mRNA levels of PHA-plus-PMA-stimulated Jurkat cells and of the permanently transfected Jurkat cell line 19D are shown also; the latter cell line constitutively produces low but detectable levels of Tax (2, 72).

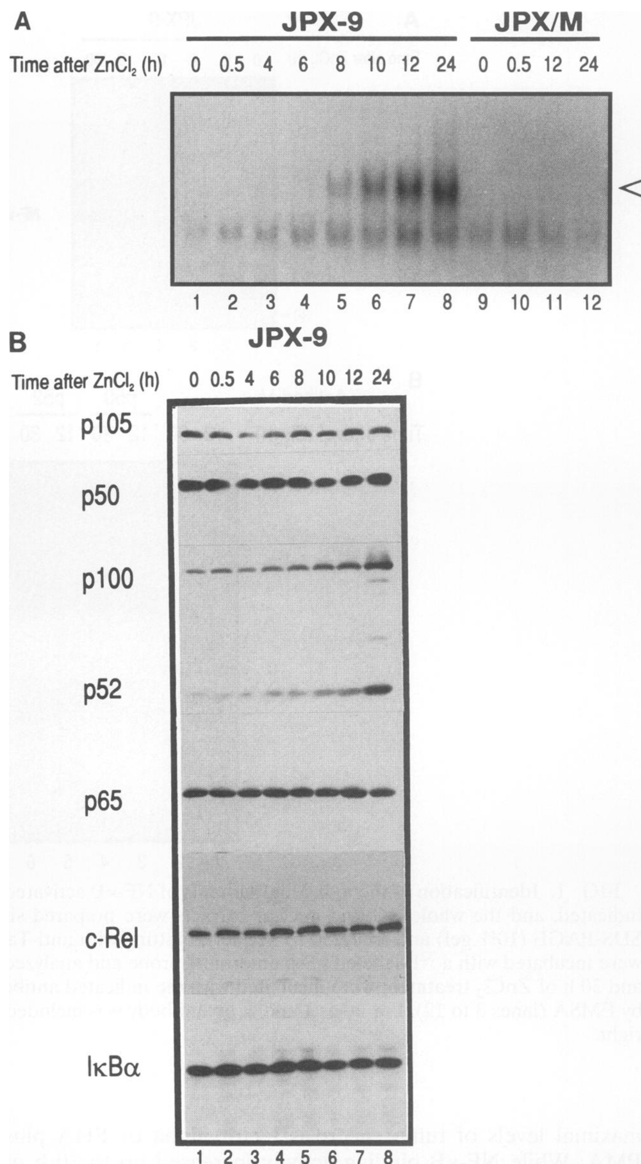

FIG. 3. Initial activation of p50/p65 heterodimers by Tax is not due to increased protein levels of p50 or p65. JPX-9 and JPX/M cells were treated with $ZnCl_2$ for the indicated periods, and whole-cell extracts were prepared. (A) The whole-cell extracts were incubated with a ^{32}P-labeled κB palindromic probe and analyzed by EMSA. The position of induced NF-κB complexes is shown by a triangle. (B) The whole-cell extracts from JPX-9 cells were separated by SDS-PAGE (10% gel) and analyzed by Western blotting using antibodies for p50/p105, p52/p100, p65, c-Rel, and IκB-α.

Analysis of the protein levels for components of NF-κB activity further supported the conclusion that the initial activation of NF-κB by Tax was independent of induced production of NF-κB itself (Fig. 3B). No changes in protein levels could be detected at the time NF-κB had been activated. In this experiment, NF-κB activation was recorded as early as 8 h after Tax induction was initiated by zinc; again this was seen only in the cells containing functional Tax but not in the cells with mutant Tax (Fig. 3A; analysis of same cells used in Fig. 3B). While levels of p50, p105, p65, c-Rel, and IκB-α had not significantly changed by 24 h, some increase in p100 and its

processed product p52 was observed at that time. Given the increase in c-Rel mRNA by 30 h, c-Rel protein levels are likely to increase at that time as well. Of note, although mRNA levels of IκB-α showed a very dramatic increase (Fig. 2), the steady-state IκB-α protein levels remained essentially unchanged at the time points analyzed.

Tax also did not accelerate the processing of the p105 precursor protein, since the ratio of p105 to p50 remained constant throughout the time course shown. This result ruled out another potential mechanism for the initial activation of NF-κB, since increased processing of the precursor within a p105/p65 cytoplasmic complex could have led to direct nuclear translocation. Further studies in which the metabolically labeled p105 and p100 precursors were subjected to a pulse-chase confirmed that Tax did not change the rate with which the precursors were processed (data not shown).

Tax induces proteolytic degradation of IκB-α. Stimulation of cells by extracellular signals is known to activate NF-κB via degradation of IκB-α (7, 15, 16, 18, 33, 67). Protein levels for IκB-α apparently did not change during Tax activation (Fig. 3B and experiments not shown that analyze shorter time intervals), raising the possibility that some Tax-mediated modification of NF-κB may have interfered with its interaction with IκB-α, thus activating the transcription factors. However, DNA binding by Tax-activated NF-κB complexes remained sensitive to inhibition by added IκB-α in cell extracts (data not shown). While IκB-α mRNA levels were dramatically upregulated by activated NF-κB (Fig. 2), the total protein levels for IκB-α remained constant, possibly indicative of a more rapid turnover of IκB-α. To investigate whether IκB-α was degraded in a Tax-dependent manner, we treated cells with the protein synthesis inhibitor cycloheximide to prevent newly synthesized IκB-α from masking the potential Tax-induced loss of preformed and complexed IκB-α (Fig. 4). Tax-induced cells, when treated with cycloheximide, showed a significant decline in existing, preformed IκB-α over a 4-h period (Fig. 4B, lanes 4 to 6), while cells with nonfunctional Tax, treated in the same fashion, revealed only minimal loss of IκB-α protein (Fig. 4C, lanes 4 to 6). Also shown are the effects of cycloheximide treatment alone for 2 and 4 h, for both JPX-9 and JPX/M cells, without zinc treatment (Fig. 4B and C, lanes 1 to 3). Figure 4A shows the corresponding NF-κB activity observed at the indicated times; the minimal amount of activation seen with cycloheximide treatment alone is likely the result of natural turnover of IκB-α. The particular time points were chosen to ensure that sufficient levels of Tax had accumulated to activate NF-κB efficiently, even in the presence of cycloheximide. The data demonstrate Tax-induced degradation of preformed IκB-α. The relatively slow loss of preformed IκB-α is consistent with a slow activation of NF-κB (see above).

We sought to confirm the Tax-mediated loss of IκB-α by an independent experimental approach which did not involve protein synthesis inhibitors (Fig. 5). JPX-9 cells were pulse-labeled for 1 h with [^{35}S]methionine and [^{35}S]cysteine prior to treatment with zinc, and whole-cell extracts were prepared at the times indicated. The labeled IκB-α proteins were detected by coimmunoprecipitation with p65-specific antibodies (Fig. 5B; the corresponding NF-κB activity is shown in Fig. 5A). This pulse-chase procedure allowed for the detection of preformed, complexed IκB-α proteins without interference by the newly synthesized IκB-α. As discussed above, activated NF-κB rapidly induces IκB-α, masking the loss of preformed IκB-α in the absence of a pulse-chase. Labeled p65 was quite stable throughout the chase of up to 12 h regardless of zinc treatment. While IκB-α declined only slowly in the cells without Tax (no zinc treatment; lanes 1 to 4), the protein disappeared significantly faster in cells with Tax (zinc treatment; lanes 5 to 7). Similar results were obtained by coimmunoprecipitation with p50-specific antibodies and IκB-α-specific antibodies (data not shown). Equal loading of lanes is indicated by the background, which did not change between 6 and 12 h postchase. The data agree with those of the previous experiment in which new IκB-α synthesis was blocked with cycloheximide. Tax-mediated activation of NF-κB involved accelerated degradation of IκB-α and loss from p65 in a manner consistent with the appearance of activated NF-κB in the nucleus.

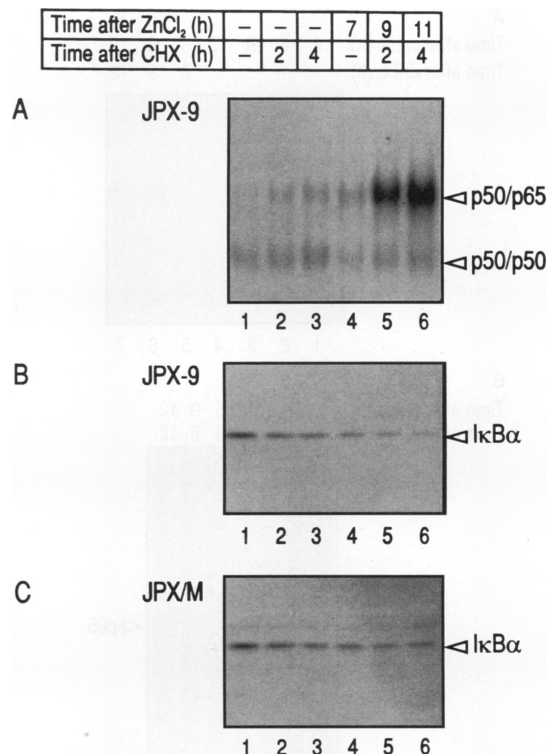

FIG. 4. Tax-induced proteolytic degradation of IκB-α. JPX-9 and JPX/M cells were treated with $ZnCl_2$ for the indicated periods (lanes 4 to 6). After 7 h of $ZnCl_2$ treatment, 10 μg of cycloheximide (CHX) per ml was added (for lanes 5 and 6). As controls, the cells not treated with $ZnCl_2$ were incubated with cycloheximide for 2 h (lane 2) and 4 h (lane 3). (A) The whole-cell extracts from JPX-9 cells were incubated with a ^{32}P-labeled κB palindromic probe and analyzed by EMSA. (B and C) Whole-cell extracts from JPX-9 (B) and JPX/M (C) cells were separated by SDS-PAGE (12% gel) and analyzed by Western blotting using an anti-IκB-α antibody. In panel B, IκB-α levels at 2 h (lane 2) and 4 h (lane 3) of cycloheximide treatment were reduced by 35 and 51%, respectively, compared with untreated cell levels (lane 1), while treatment of $ZnCl_2$-treated cells for 2 h (lane 5) and 4 h (lane 6) with cycloheximide resulted in reductions by 65 and 91%, respectively, when compared with levels prior to cycloheximide addition (lane 4) (values were determined by densitometry).

DISCUSSION

We investigated potential mechanisms by which the HTLV-I Tax protein may activate the transcription factor complexes collectively referred to as NF-κB. We provide direct physical evidence, for the first time, that the Tax-mediated activation involves the induced degradation of the NF-κB-inhibitory protein IκB-α. Degradation is also induced during physiological stimulation by extracellular agents and is a necessary step in the activation of NF-κB (7, 15, 16, 18, 33, 67). Tax may

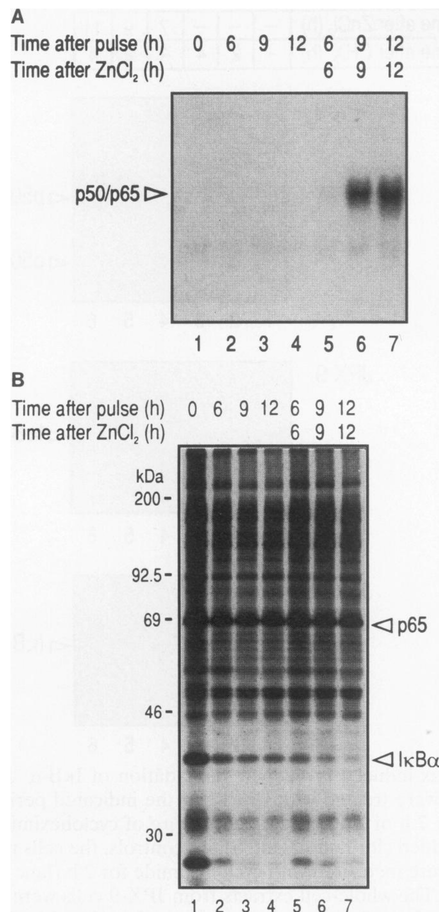

FIG. 5. Tax-induced loss of p65-coupled IκB-α. JPX-9 cells were metabolically labeled and divided into two groups. One group was treated with $ZnCl_2$ (lanes 5 to 7), and the other was untreated (lanes 1 to 4). Whole-cell extracts were prepared at the indicated time points and incubated with a ^{32}P-labeled κB palindromic probe for EMSA (A) or coimmunoprecipitated with an anti-p65 antibody (B). (A) To detect signals only from the ^{32}P-labeled probe, two X-ray films were exposed simultaneously, and only the shielded second autoradiogram is shown. (B) The precipitated complexes were separated by SDS-PAGE (10% gel) and visualized by fluorography. The positions of IκB-α and p65 are indicated. Levels of p65-complexed IκB-α at 9 h (lane 3) and 12 h (lane 4) after pulse-labeling were reduced by 32 and 38%, respectively, compared with levels at 6 h (lane 2), while induction of Tax by $ZnCl_2$ treatment resulted in reductions by 64% at 9 h (lane 6) and 92% at 12 h (lane 7) compared with levels at 6 h (lane 5) (values were determined by densitometry).

function in the signal transduction pathway(s) upstream of the final target IκB-α, or it may function at the NF-κB/IκB-α level. Tax therefore mediates its effects in the cytoplasm, consistent with both cytoplasmic and nuclear localization of this protein (27, 38). The Tax-induced rapid turnover of IκB-α may weaken the effectiveness of the inhibitor; it may be responsible for the initial activation of NF-κB and may also allow continuous translocation of NF-κB complexes into nuclei, despite normal amounts of IκB-α due to increased resynthesis.

While previous studies have focused largely on cells which constitutively synthesized Tax (2, 4, 40, 44) or which were transiently transfected to express Tax (62), we analyzed an inducible Jurkat cell system in which Tax expression was entirely dependent on treatment of cells with low levels of $ZnCl_2$ (50, 55). Activation of NF-κB by induced Tax expression has also been demonstrated previously (44). In the present study, strong activation of NF-κB was due entirely to the induced Tax protein rather than to the treatment with metal itself. Throughout the study, we made use of control cells in which a mutant, nonfunctional Tax protein was induced with zinc. This Tax-inducible system allowed a careful kinetic analysis of the activation by this HTLV-I-encoded protein. We established that Tax did not activate NF-κB by first upregulating the synthesis of component subunits of NF-κB, a hypothesis considered previously (2, 44). At the time p50/p65 heterodimers were already activated, the level of mRNAs or protein for the component parts had not changed relative to those of untreated cells. That Tax functions posttranslationally is consistent with a prior report in which NF-κB was activated in cells to which recombinant Tax was added in the presence of protein synthesis inhibitors, assuming that the extracellularly applied Tax did not generate a signal from outside the cells (46). Subsequent to the initial activation by Tax, however, the levels of several mRNAs and proteins did increase, in particular those for p52 and c-Rel, possibly serving as the basis for the later increase of activated complexes containing these proteins. Higher levels of p52 and c-Rel proteins have recently been reported for the HTLV-I-infected and Tax-expressing cell-line MT2 (41).

The appearance, first, of p50/p65 heterodimers in the nucleus and then, later, a number of other dimers mirrors what occurs with extracellular stimulation of Jurkat cells as well (49). This similarity in the responses to very different agents may reflect a common underlying mechanism; certainly the degradation of IκB-α is common to both Tax action and signaling from outside the cell. The two responses do differ kinetically, however, since Tax-mediated activation of the early complexes is slow compared with activation with extracellular signals; a few hours versus minutes. Since Tax accumulates during the activation phase, this may simply reflect a necessary threshold level required of this protein, or it may herald more complex actions of Tax. Another unique feature of the Tax-mediated activation is the strong induction of p52/p100 mRNA and protein relative to the induction of p50/p105.

Our data on the levels of the p105 and p100 precursor proteins vis-à-vis their processed forms p50 and p52 exclude another potential mechanism for Tax activity. Recently, both the p100 and p105 precursor molecules have been implicated as NF-κB inhibitors as a result of the presence of IκB-α-like ankyrin domains in their C-terminal halves (47, 52, 53, 56, 60, 68). The precursors can thus be envisioned to retain heterodimerized p65 or c-Rel in the cytoplasm without involvement of IκB-α. Theoretically, accelerated processing of the precursor molecules could have caused activation by the rapid conversion of, e.g., p105/p65 complexes into high levels of p50/p65 heterodimers, which in turn might have overwhelmed the capacity of the IκB-α inhibitor. However, no change in the rate of processing of the precursors was recorded in the presence of Tax. Furthermore, pulse-chase experiments in which the metabolically labeled precursors were chased into their processed forms failed to reveal an effect of Tax on processing.

The proteolytic degradation of IκB-α is a necessary step in the activation of NF-κB by extracellular signals (7, 15, 16, 18, 33, 67). We demonstrate here by two independent experimental approaches that Tax significantly accelerates loss of IκB-α as well, albeit slowly compared with normal signaling. The relatively slow degradation correlates well with the delayed and gradual activation of NF-κB. With both methods, we were able to visualize specifically those IκB-α proteins which had been

synthesized some time before and/or which were bound to NF-κB complexes, avoiding complications due to newly synthesized IκB-α proteins. The importance of the proteolytic degradation of IκB-α for at least initial activation of NF-κB by Tax was further suggested by experiments involving protease inhibitors (data not shown). Protease inhibitors such as tosyl phenylalanine chloromethyl ketone (TPCK) are known to block activation of NF-κB by extracellular signals, correlating with the continued presence of the IκB-α inhibitor (33). Tax activation of NF-κB was blocked by TPCK as well, but the experiment was complicated by the toxic side effects of TPCK during the relatively long exposure time needed. Finally, the possibility that Tax mimics a normal signal pathway to activate NF-κB was revealed in experiments in which a phosphorylated form of IκB-α could be detected, albeit at very low levels (data not shown). The phosphorylated form appears to be an immediate target for the IκB-α protease(s), making the detection of this modified and only very transiently present form difficult even with a rapid and strong activation signal (7, 15, 18, 68). The slow activation by Tax may result in only barely detectable levels of the modified form. The involvement of signaling components in Tax function is consistent also with a previous report in which Tax-mediated activation of NF-κB was partially blocked in the presence of the ion chelator PDTC, which is known to block the generation of reactive oxygen intermediates and thus block messengers which have been suggested to be important for signaling to NF-κB with extracellular stimuli (62). However, the fate of IκB-α was not examined in that study.

Given our data, we speculate that Tax-induced degradation of IκB-α triggers the initial translocation of NF-κB into the nucleus. As a result, strong induction of IκB-α synthesis ensues, driven by the transcriptional activator NF-κB itself (15, 16, 18, 19, 42, 63, 67). In spite of increased resynthesis of IκB-α, NF-κB activation persists, a situation which may be analogous to that of mature B cells. Such cells display constitutive NF-κB activity and rapid turnover of IκB-α (48). Possibly, the Tax-induced ongoing degradation of complexed IκB-α allows at least some NF-κB complexes to continually escape into the nucleus before newly synthesized IκB-α can recapture it. The free IκB-α itself is very unstable, leading to a steady state in which the total level of IκB-α is maintained but in which the pool of these molecules turns over rapidly because of Tax. The rapid turnover thus interferes with efficient inhibition. The present model does not exclude other, additional mechanisms by which Tax may activate NF-κB. As discussed above, further functions of Tax may be required to activate the later-appearing complexes. In experiments which go beyond the scope of this study, we have recently discovered that Tax may indeed have additional activities (37) which are likely to be mediated by association with the p100 precursor (9). Nonetheless, the molecular details of the initial activation of NF-κB by Tax fit well with a presumed role of Tax in activating a component of an intrinsic signaling cascade, targeting for degradation the inhibitory IκB-α protein.

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