Human T-Cell Leukemia Virus Type I Tax Activation of NF- κ B/Rel Involves Phosphorylation and Degradation of I κ B α and RelA (p65)-Mediated Induction of the *c-rel* Gene

SHAO-CONG SUN, JOHN ELWOOD, CHRISTOPHE BÉRAUD, AND WARNER C. GREENE*

Gladstone Institute of Virology and Immunology, University of California, San Francisco, and
San Francisco General Hospital, San Francisco, California 94141-9100

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The *tax* gene product of human T-cell leukemia virus type I (HTLV-I) is a potent transcriptional activator that both stimulates viral gene expression and activates an array of cellular genes involved in T-cell growth. Tax acts indirectly by inducing or modifying the action of various host transcription factors, including members of the NF- κ B/Rel family of enhancer-binding proteins. In resting T cells, many of these NF- κ B/Rel factors are sequestered in the cytoplasm by various ankyrin-rich inhibitory proteins, including I κ B α . HTLV-I Tax expression leads to the constitutive nuclear expression of biologically active NF- κ B and c-Rel complexes; however, the biochemical mechanism(s) underlying this response remains poorly understood. In this study, we demonstrate that Tax-stimulated nuclear expression of NF- κ B in both HTLV-I-infected and Tax-transfected human T cells is associated with the phosphorylation and rapid proteolytic degradation of I κ B α . In contrast to prior *in vitro* studies, at least a fraction of the phosphorylated form of I κ B α remains physically associated with the NF- κ B complex *in vivo* but is subject to rapid degradation, thereby promoting the nuclear translocation of the active NF- κ B complex. We further demonstrate that Tax induction of nuclear c-Rel expression is activated by the RelA (p65) subunit of NF- κ B, which activates transcription of the *c-rel* gene through an intrinsic κ B enhancer element. In normal cells, the subsequent accumulation of nuclear c-Rel acts to inhibit its own continued production, indicating the presence of an autoregulatory loop. However, the pathologic action of HTLV-I Tax leads to the deregulated and sustained nuclear expression of both NF- κ B and c-Rel, a response that may contribute to HTLV-I-induced T-cell transformation.

Human T-cell leukemia virus type I (HTLV-I) is a type C retrovirus that primarily produces disease within the CD4⁺ subset of human T cells (for recent reviews, see references 27 and 88). Infection with HTLV-I has been etiologically linked with an aggressive CD4⁺ T-cell malignancy termed adult T-cell leukemia (61, 89) and several different nonmalignant diseases (27, 88), including a chronic neurodegenerative syndrome termed tropical spastic paraparesis (30) or HTLV-I-associated myelopathy (60). Although the precise mechanism by which HTLV-I transforms human T cells remains unclear, the HTLV-I transactivator protein, Tax, likely plays a central role. Indeed, Tax has been shown to possess transforming potential both in cultured cells (62, 75, 82) and in transgenic mice (39, 57).

Tax functions as a potent transcriptional activator that induces the expression of all viral genes controlled by the HTLV-I long terminal repeat (LTR) (21, 76) as well as various cellular genes (1, 24, 28, 44, 51, 55, 73), many of which are involved in T-cell activation and growth. These latter cellular genes include the interleukin-2 (IL-2) gene (51, 73) and the gene encoding the alpha chain of IL-2 receptor (IL-2R α) (24, 44, 73). The deregulated expression of these growth-related cellular genes induced by Tax likely contributes to the polyclonal proliferation of T cells observed early after HTLV-I infection (34). This proliferative process may in turn facilitate the occurrence of subsequent undefined genetic events that

complete the transformation process leading clinically to the adult T-cell leukemia.

Tax does not bind directly to DNA. Rather, Tax appears to alter the target gene expression in a more indirect manner by modulating the activity or expression of specific host transcription factors (3, 35). For example, Tax activates the HTLV-I LTR by physical interaction with HEB-1 or the CREB/ATF family of proteins, which specifically bind to the 21-bp repeats of the HTLV-I LTR (15, 81, 90, 91). A recent study suggests that Tax may also act by enhancing the dimerization and subsequent binding of various CREB factors (84). Induction of the IL-2 and IL-2R α genes by Tax involves the activation of nuclear expression of the NF- κ B/Rel family of transcription factors (11, 23, 43, 65).

The NF- κ B/Rel family of transcription factors includes the p50 and RelA (previously termed p65) subunits of the prototypical NF- κ B complex (8, 12, 17, 32, 47, 53, 59, 66, 72), p52 (18, 58, 68), the viral oncoprotein v-Rel (77, 87), the cellular factor c-Rel (20), the *dorsal* gene product of *Drosophila melanogaster* (78), and a number of other structurally related DNA-binding proteins (33). In resting T cells, most of the NF- κ B/Rel proteins are sequestered in the cytoplasm as latent precursors by a family of ankyrin-rich inhibitory proteins that include I κ B α (5–7, 14, 29, 37), p105 (product of the NF- κ B1 gene [52, 56, 64]), and p100 (product of the NF- κ B2 gene [52, 56, 80]). I κ B α appears to play a major regulatory role in governing the immediate-early expression of the NF- κ B p50/RelA heterodimer that accompanies cellular activation. Specifically, such cellular activation leads to the rapid proteolytic degradation of this cytoplasmic inhibitor, a process which in turn promotes the nuclear translocation of the NF- κ B heterodimer (13, 19, 22, 38, 63, 79). Although small amounts of

* Corresponding author. Mailing address: Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94141-9100. Phone: (415) 826-7500. Fax: (415) 826-1817. Electronic mail address: Warner_Greene@quickmail.ucsf.edu.

the c-Rel protein are similarly sequestered in the cytoplasm of resting T cells, the predominant portion of nuclear c-Rel expression is the result of de novo protein synthesis and thus temporally delayed compared with NF- κ B (54). At present, the precise mechanism by which c-Rel protein expression is induced is not well understood.

Similarly, although the HTLV-I Tax protein is known to strongly activate nuclear expression of both NF- κ B and c-Rel (3, 74), the underlying mechanism by which Tax produces these effects remains unclear. Tax has been shown to physically associate with p105 (40) and even more strongly with p100 (16, 49). However, in our hands the interaction of p100 with Tax leads to an inhibition of Tax-mediated transactivation (16) rather than participating in the induction of nuclear transcription factor expression by promoting the cleavage of p100 to p52. In contrast, Watanabe and colleagues have recently proposed that Tax stimulates the release of p105 from p50 and RelA, thereby allowing nuclear translocation of the active NF- κ B complex (86).

In this report, we demonstrate that Tax-mediated activation of NF- κ B in both HTLV-I-infected and Tax-transfected T cells involves the phosphorylation of I κ B α . Although the phosphorylated form of I κ B α remains physically associated with NF- κ B, this modified inhibitor is targeted for rapid degradation, thereby allowing NF- κ B to translocate to the nucleus. We further show that the rapidly induced nuclear expression of NF- κ B triggers transcription of the *c-rel* proto-oncogene, through a functional 5' κ B enhancer element. These findings demonstrate that one Rel-related protein can control the expression of a second Rel protein. Finally, we show that the accumulation of c-Rel normally serves to inhibit its own further production; however, HTLV-I Tax is capable of overriding this feedback inhibitory loop, leading to sustained high-level nuclear expression of both NF- κ B and c-Rel.

MATERIALS AND METHODS

Cell lines. The HTLV-I-infected T-cell lines C8166, HUT 102, and K3T as well as the noninfected Jurkat leukemic T cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Jurkat-Tax and Jurkat-anti-Tax cells (85) were cultured in the same medium containing 800 μ g of the antibiotic G418 per ml to select for expression of the neomycin resistance gene. F9 embryonal carcinoma cells were cultured in Iscove's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

Immunoblotting, immunoprecipitation, and electrophoresis mobility shift assay (EMSA). The HTLV-I-infected T cells and noninfected Jurkat T cells were collected by centrifugation at $800 \times g$ for 5 min. Subcellular protein extracts were prepared as previously described (70) except that the cell lysis buffer was supplemented with various phosphatase inhibitors, including 10 mM NaF, 0.1 mM Na₃VO₄, 2 mM EDTA, 50 μ M ZnCl₂, and 10 μ M sodium molybdate (80). The protein extracts (500 μ g) were subjected to immunoprecipitation as described previously (29), and the immunoprecipitated proteins were analyzed by immunoblotting as described below.

For immunoblotting analyses, protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with various antipeptide antisera, using an enhanced chemiluminescence detection system (Amersham). For phosphatase treatment, the extracts were incubated with calf intestinal alkaline phos-

phatase (0.15 U/ μ g of extract) or incipient buffer controls for 30 min at 37°C prior to immunoblotting analysis.

EMSAs were performed by incubating the nuclear extracts (5 μ g) with a ³²P-radiolabeled high-affinity palindromic κ B probe, κ B-pd (coding strand sequence was 5'-CAACGGCAG GGGAATCCCCCTCTCCTT-3'), and then resolving the DNA-protein complexes on native 5% polyacrylamide gels (10).

Plasmids, transient transfection, and luciferase assays. All cDNAs were subcloned into the pCMV4 expression vector generously provided by M. Stinski and D. Russell (2). N- and C-terminal deletion mutants of RelA were generated by PCR using specific primers and the full-length RelA cDNA as a template (29). p65(1-312) corresponds to a C-terminal truncation mutant of RelA that retains the N-terminal Rel homology domain but lacks the entire C-terminal transactivation domain of RelA. This Rel mutant functions as a transdominant inhibitor of NF- κ B. p65(41-551) is an N-terminal truncation mutant that lacks the N-terminal 40 amino acids and is defective in DNA binding (29). The reporter plasmids pLuc-97 and pLuc-23 (a kind gift from the late Howard Temin) were constructed by inserting the chicken *c-rel* promoter upstream of the luciferase reporter gene (36). pLuc-97 retains a κ B enhancer element, GGGAATTTCCC, located at -28 which is partially deleted in the pLuc-23 plasmid.

Jurkat cells (5×10^6) were transfected by using DEAE-dextran (42) with 2 μ g of pLuc-97 or pLuc-23 together with the indicated amount of *tax* or transdominant *relA* cDNA expression vector. F9 cells were seeded onto 0.1% gelatin-treated, six-well plates (3×10^5 cells per well) 1 day before transfection. pLuc-97 (0.5 μ g) and the indicated amounts of the various cDNA expression vectors were transfected by using the Lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. Cell extracts were normalized for protein recovery (Bio-Rad) and assayed for luciferase activity (Promega) 40 to 48 h after transfection.

RESULTS

HTLV-I Tax expression is correlated with the constitutive phosphorylation of I κ B α and sustained nuclear expression of RelA and c-Rel. The pattern of I κ B α protein expression was analyzed by immunoblotting extracts from long-term HTLV-I-infected T-cell lines (C8166, HUT102, and K3T) or human Jurkat T cells stably transfected with either a functional sense (Jurkat-Tax) or nonfunctional antisense (Jurkat-anti-Tax) *tax* cDNA expression plasmid (85) (Fig. 1A). In both Jurkat-anti-Tax cells and control untransfected parental Jurkat cells, only a single 37-kDa form of I κ B α was detected with an anti-I κ B α antiserum (lanes 1 and 6). In contrast, a second, more slowly migrating I κ B α species was present in each of the Tax-expressing HTLV-I cell lines (lanes 2 to 4) and in smaller amounts in the Jurkat-Tax cells (lane 5). Expression of this apparently larger I κ B α species was correlated not only with Tax expression (Fig. 1B) but also with nuclear expression of NF- κ B/Rel DNA-binding activity, as measured in EMSA (Fig. 1C). The slower mobility of this I κ B α species was apparently the result of phosphorylation, as this form could be completely converted to the faster-migrating 37-kDa species by in vitro treatment with calf intestinal alkaline phosphatase (Fig. 1D, lanes 3 and 4) but not by the incipient buffer control (lanes 1 and 2).

Parallel immunoblotting studies of cytoplasmic and nuclear fractions from these cells revealed that both c-Rel (Fig. 2A) and the RelA subunit of NF- κ B (Fig. 2B) were constitutively expressed in the nuclei of these Tax-expressing cells (lanes 4, 6, 8, and 10). In contrast, in parental Jurkat (lane 2) and

FIG. 1. Correlation between phosphorylation of I κ B α and expression of nuclear NF- κ B/Rel DNA-binding activity in HTLV-I-infected or Tax-expressing human T cells. (A and B) A modified form of I κ B α is present in Tax-expressing cells. Cytoplasmic (A) or whole-cell (B) extracts (80 μ g) were prepared from the indicated cells and subjected to immunoblotting with peptide-specific antisera directed against either the C terminus (amino acids 289 to 317) of I κ B α (A) or the C terminus (amino acids 321 to 353) of Tax (B). (C) Nuclear NF- κ B/Rel DNA-binding activity is correlated with Tax expression and the appearance of the modified form of I κ B α . Nuclear extracts (5 μ g) were analyzed by EMSA for the presence of NF- κ B/Rel DNA-binding activity, using a 32 P-radiolabeled κ B probe (see Materials and Methods). (D) The modified form of I κ B α is phosphorylated. Cytoplasmic extracts from C8166 cells (lanes 1 and 3) or HUT102 cells (lanes 2 and 4) were incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of calf intestinal alkaline phosphatase (CIP) and then subjected to immunoblotting analysis with anti-I κ B α .

Jurkat-anti-Tax (lane 12) cells, these factors were principally confined to the cytoplasm. Additionally, on a whole-cell basis, the overall level of c-Rel expression appeared slightly greater in the Tax-expressing cells than in the Jurkat cell controls (Fig. 2A).

Phosphorylated I κ B α remains associated with RelA but is subject to rapid degradation. To further explore the biochemical basis for induction of NF- κ B and c-Rel in the HTLV-I-infected cells, studies were performed to test whether phosphorylation of I κ B α resulted in its physical separation from RelA and c-Rel (Fig. 3A). In surprising contrast to the

FIG. 2. Subcellular pattern of expression of c-Rel and RelA in various HTLV-I-infected cells and Jurkat-Tax or Jurkat anti-Tax cell lines. Cytoplasmic (C) and nuclear (N) extracts (20 μ g) were subjected to immunoblotting with antisera specific for either the C terminus (amino acids 573 to 587) of c-Rel (A) or the C terminus (amino acids 529 to 551) of Rel A (B).

FIG. 3. Effect of I κ B α phosphorylation on its physical association with Rel A and c-Rel and subsequent intracellular survival. (A) Physical association of phosphorylated I κ B α with RelA and c-Rel. Cytoplasmic extracts from HUT102 cells were immunoprecipitated with either a preimmune serum (lane 4) or a peptide-specific anti-serum recognizing I κ B α (lane 1), RelA (lane 2), or c-Rel (lane 3). The resultant immunoprecipitates were subjected to immunoblotting with an I κ B α -specific antiserum. The positions of migration of I κ B α and its phosphorylated isoform (I κ B α -P) are indicated. (B) Intracellular fate of I κ B α and I κ B α -P in Tax-expressing C8166 and Tax-nonexpressing Jurkat T cells. C8166 cells or Jurkat cells were incubated in the presence of CHX (50 μ g/ml) for the indicated time intervals and then subjected to immunoblotting analysis of the expression of I κ B α and I κ B α -P present in the whole-cell extracts.

complete release of I κ B α from NF- κ B proposed on the basis of in vitro phosphorylation experiments (31), both phosphorylated and nonphosphorylated forms of I κ B α from HUT102 cells could be detected in physical association with RelA, as judged from their coimmunoprecipitation with RelA with use of RelA-specific antibodies (lane 2). Similarly, anti-c-Rel coimmunoprecipitated phosphorylated I κ B α , although the overall recovery of I κ B α species was diminished with the anti-c-Rel antibody (lane 3).

To investigate whether phosphorylation of I κ B α altered its intracellular survival, the fate of both the phosphorylated and 37-kDa forms of I κ B α was examined in unstimulated Jurkat T cells and Tax-expressing HTLV-I-infected T cells treated with cycloheximide (CHX) to block de novo synthesis of this inhibitor. As shown in lanes 7 and 8 of Fig. 3B, essentially no change in the level of expression of the 37-kDa I κ B α species occurred over a 2-h period following CHX treatment of Jurkat cells. These findings indicate a relatively long half-life ($t_{1/2}$ > 120 min) for this unmodified form of I κ B α in these noninfected T cells. In contrast, the phosphorylated I κ B α species (I κ B α -P) present in the Tax-expressing C8166 cells proved quite unstable, exhibiting a $t_{1/2}$ of less than 5 min in the presence of CHX (lanes 1 to 6, upper band). The unmodified 37-kDa I κ B α species was also rapidly degraded in these cells, likely reflecting ongoing phosphorylation of I κ B α induced by the previously synthesized Tax protein that remained active in these cells. Similar results were obtained in independent experiments using two other Tax-expressing HTLV-I-infected T-cell lines (data not shown). Together, these findings suggest that the phosphorylation of I κ B α occurring in HTLV-I-infected cells likely targets this inhibitor for rapid intracellular degradation.

Inhibition of NF- κ B nuclear expression by chymotrypsin-type protease inhibitors is correlated with the blockade of I κ B α phosphorylation and degradation. To investigate the potential role of various types of proteases in the intracellular destabilization of I κ B α , the effects of different protease inhibitors were studied. As shown in Fig. 4, treatment of Tax-expressing C8166 cells for 4 h with the chymotrypsin-type protease inhibitor tosylphenylalanine chloromethyl ketone

FIG. 4. Effect of the chymotrypsin-type TPCK and trypsin-type TLCK protease inhibitors on nuclear NF- κ B/Rel DNA-binding activity in HTLV-I-infected T cells. C8166 cells were either not treated or incubated with 25 or 50 μ M TPCK or TLCK for 4 h at 37°C, after which nuclear NF- κ B/Rel DNA-binding activity was analyzed by EMSA.

(TPCK) partially (25 μ M) or completely (50 μ M) inhibited nuclear expression of NF- κ B/Rel (lanes 2 and 3). In contrast, the trypsin-type protease inhibitor tosyllysine chloromethyl ketone (TLCK) exerted no significant inhibitory effects (lanes 4 and 5). Of note, TPCK has previously been shown to inhibit T-cell activation by blocking early T-cell signaling events, including calcium mobilization (4). Accordingly, we considered the possibility that this protease inhibitor acts by inhibiting a more proximal signal transduction event leading to the phosphorylation of I κ B α rather than simply blocking subsequent protease-mediated degradation of I κ B α . Consistent with this notion, incubation of C8166 cells with TPCK resulted in the rapid disappearance of phosphorylated I κ B α accompanied by accumulation of the 37-kDa nonphosphorylated I κ B α species (Fig. 5A, lanes 2 to 4). Indeed, within 15 min after TPCK addition, almost the entire intracellular pool of I κ B α corresponded to the unphosphorylated 37-kDa form of I κ B α (Fig. 5A, lanes 3 and 4). To investigate whether this apparent blockade of phosphorylation of I κ B α mediated by TPCK in turn produced intracellular stabilization of this inhibitor, CHX was added to cells preincubated with TPCK for 1 min (lane 6) or 2 h (lane 5). When present for 2 h, TPCK blocked the rapid degradation of I κ B α seen in the presence of CHX alone (compare lanes 5 and 9), which was correlated with conversion of the entire I κ B α pool to its nonphosphorylated form (lane 4). Consistent with its inability to inhibit nuclear expression of NF- κ B/Rel in these HTLV-I-infected cells, TLCK neither inhibited the phosphorylation of I κ B α (lane 7) nor prevented the rapid degradation of this inhibitor observed in the presence of CHX (lane 8).

To further explore the relationship between the phosphorylation and degradation of I κ B α , the effects of TPCK were next examined on tumor necrosis factor alpha (TNF- α)-induced phosphorylation of I κ B α . In agreement with prior reports (13, 80), TNF- α transiently induced phosphorylation of I κ B α in Jurkat cells (Fig. 5B, lanes 2 and 3). However, addition of TPCK for 5 min prior to TNF- α addition resulted in a complete inhibition of this induced phosphorylation (lanes 5 and 6), while addition of TLCK was without effect (lanes 8 and 9). Similar inhibition of both phosphorylation and degradation of I κ B α induced by Tax or TNF- α was observed following the addition of pyrrolidinedithiocarbamate (PDTC) (data not shown), a free-radical scavenger and metal chelator previously reported to block NF- κ B induction (69). Together, these findings are most consistent with Tax- or TNF- α -induced phosphorylation of I κ B α serving as a molecular trigger that leads to the subsequent rapid degradation of this inhibitor and nuclear translocation of NF- κ B and c-Rel.

The c-rel proto-oncogene is transcriptionally induced by

FIG. 5. Effects of TPCK and TLCK on the phosphorylation and intracellular stability of I κ B α . (A) Effects of TPCK and TLCK on the phosphorylation and degradation of I κ B α in HTLV-I-infected C8166 cells. C8166 cells were either untreated (lane 1) or incubated with 50 μ M TPCK (lanes 2 to 6) or 50 μ M TLCK (lanes 7 and 8) for the indicated time periods. Aliquots of these cells were further incubated with 50 μ g of CHX per ml (lanes 5, 6, 8, and 9) for the indicated time periods to analyze I κ B α survival in the absence of new protein synthesis. Whole-cell extracts were then analyzed for the presence of I κ B α and I κ B α -P by immunoblotting. (B) Effects of TPCK and TLCK on TNF- α -induced phosphorylation and degradation of I κ B α in Jurkat T cells. These cells were either untreated (lanes 1 to 3) or cultured with 50 μ M TPCK (lanes 4 to 6) or TLCK (lanes 7 to 9) for 5 min prior to stimulation with TNF- α (10 ng/ml) for the indicated time periods, after which I κ B α and I κ B α -P protein expression was analyzed by immunoblotting.

RelA but autorepressed by the c-Rel protein. Prior studies have demonstrated that nuclear expression of the p50 and RelA subunits of NF- κ B occurs within minutes in Jurkat T cells induced with either phorbol 12-myristate 13-acetate (PMA) or TNF- α (26, 54). In contrast, nuclear expression of c-Rel does not occur until several hours after PMA stimulation and appears dependent upon de novo protein synthesis (54). In Tax-expressing T cells, both NF- κ B and c-Rel are constitutively expressed at high levels in the nucleus. In view of these findings, we investigated the possibility that Tax-induced c-Rel expression is activated by NF- κ B. For these studies, Jurkat T cells were transfected with a Tax cDNA expression vector alone or in combination with increasing amounts of a transdominant RelA mutant containing only a DNA-binding domain, p65(1-312) (29). In addition, these same cells were transfected with a chicken c-rel promoter-luciferase reporter plasmid either containing (pLuc-97) or lacking (pLuc-23) an upstream κ B enhancer (36). In agreement with a prior report (50), Tax potently activated the pLuc-97 reporter plasmid (Fig. 6A). More importantly, inclusion of the RelA transdominant mutant, p65(1-312), repressed these stimulatory effects of Tax in a dose-related manner. Tax failed to induce the pLuc-23 plasmid, which lacked a κ B enhancer element.

To independently test whether RelA regulates activation of the c-rel gene and to examine whether the c-Rel protein exerts any modulatory effects on its own expression, cotransfection experiments were performed in undifferentiated F9 embryonal

FIG. 6. Transactivation of the *c-rel* promoter by HTLV-I Tax and RelA. (A) Transactivation of the *c-rel* promoter by Tax in Jurkat T cells. Jurkat cells were transfected with either the parental pCMV4 vector lacking a cDNA insert or the indicated amount of pCMV4-based expression vector encoding Tax or a transdominant deletion mutant of p65 (RelA) lacking the C-terminal RelA activation domain, p65(1-312), together with a chicken *c-rel* promoter-luciferase reporter plasmid either containing (pLuc-97) or lacking (pLuc-23) an intrinsic κ B enhancer element. Transcriptional activation of these plasmids by Tax in the presence of various amounts of the transdominant p65(1-312) mutant was determined by assay of luciferase enzyme production (Promega luciferase assay system). (B) Effects of c-Rel and p65 (RelA) mutants on the activity of the *c-rel* promoter in undifferentiated F9 embryonal carcinoma cells. F9 cells, which lack inducible NF- κ B activity, were transfected with either the parental vector pCMV4 (V) or a cDNA expression vector encoding a Tax, c-Rel, p65 (RelA), or p65 (RelA) deletion mutant (29) lacking either the transactivation (1-312) or DNA-binding (41-551) domains together with the pLuc-97 or pLuc-23 reporter plasmid as indicated.

cells, which lack inducible NF- κ B activity (Fig. 6B). Tax alone failed to transactivate the *c-rel* promoter in these cells; however, transfection of a RelA expression plasmid produced a potent and dose-dependent induction of the κ B-containing *c-rel* promoter. This stimulatory action of RelA required both its DNA-binding and transactivation domains, as evidenced by the lack of effect of the p65(41-551) and p65(1-312) RelA deletion mutants, which lacked these two functional domains, respectively (29). In contrast to RelA, transfection of a *c-rel* expression vector did not lead to appreciable stimulation of the *c-rel* promoter. However, when coexpressed with RelA, c-Rel significantly repressed activation of the *c-rel* promoter mediated through RelA. Together, these findings indicate that the rapidly expressed NF- κ B complex containing RelA activates transcription of the *c-rel* proto-oncogene. In turn, the *c-rel* gene is subject to end-product inhibition by the subsequent accumulation of nuclear c-Rel protein.

DISCUSSION

The HTLV-I *tax* gene product is a potent intracellular inducer of nuclear NF- κ B and c-Rel expression (3, 74). In turn, these Rel-related transcription factors regulate the expression of a number of cellular genes involved in T-cell growth. The deregulated expression of these genes may contribute to T-cell transformation induced by HTLV-I. Indeed, antisense oligonucleotides to NF- κ B have recently been shown to inhibit the growth of HTLV-I Tax-induced tumor cells (48). Despite this apparent important functional interplay between Tax and NF- κ B/Rel, the underlying mechanism by which this viral transactivator induces the nuclear expression of these host transcription factors has remained elusive. In this regard, recent studies have demonstrated that Tax physically associates with the NF- κ B1 and NF- κ B2 gene products, p105 and p100 (16, 40), which are precursors of p50 and p52, respectively (17, 18, 32, 47, 53, 58, 68). Using a transient transfection model, Watanabe et al. have reported that the physical interaction of Tax may override the cytoplasmic sequestration of p50 and p65 by p105 which functions like an I κ B molecule (86).

Similarly, Hirai and colleagues have recently demonstrated a physical interaction of Tax with the ankyrin repeats present in the C-terminal portion of p105 (41), a region proposed to encode I κ B γ (45). The authors propose that this interaction may promote nuclear translocation of RelA. Unfortunately, the relevance of these findings is uncertain since I κ B γ has not yet been identified in human cells.

In contrast to these results with p105, our recent studies of the physical interplay of Tax and p100 have revealed a very different functional consequence. Specifically, the association of Tax with p100 leads to the cytoplasmic sequestration of this transactivator and marked inhibition of its inductive effects on both the HTLV-I LTR and human immunodeficiency virus type 1 κ B enhancer (16). As such, these inhibitory effects of p100 on Tax may contribute to the profound state of viral latency that characterizes adult T-cell leukemia (83). In contrast, we have assembled no evidence indicating that Tax association with p100 is involved in the activation of NF- κ B or related complexes. Specifically, in our hands, Tax fails to induce the processing of p100 to p52 and does not lead to the physical dissociation of p100 from RelA (14a). As such, the mechanism of Tax-mediated activation of NF- κ B and c-Rel remains elusive.

In this study, we have investigated the possibility that Tax-mediated activation of NF- κ B and c-Rel is mediated through the induced phosphorylation and degradation of I κ B α , the major cytoplasmic inhibitor of the RelA subunit of NF- κ B. We have in fact found that a large portion of I κ B α is phosphorylated in all three of the HTLV-I-infected T-cell lines investigated (Fig. 1A). Phosphorylation of I κ B α in these cells appears to be the result of Tax action since Tax is the only viral gene product expressed in C8166 cells (67), and similar phosphorylation of I κ B α is observed in Jurkat cells stably transfected with a sense but not antisense Tax cDNA expression vector (Fig. 1A). Additionally, the degree of I κ B α phosphorylation appears correlated with the intracellular level of Tax expression (Fig. 1).

Prior *in vitro* studies have indicated that phosphorylation of I κ B α with protein kinase C (PKC) or PKA leads to its rapid physical release from the NF- κ B complex (31). Surprisingly, our *in vivo* data have revealed that at least a portion of the phosphorylated form of I κ B α remains physically associated with RelA, as indicated by its coimmunoprecipitation with RelA (Fig. 3A). These *in vivo* findings suggest that phosphorylation of I κ B α alone may not be sufficient to activate nuclear translocation of the NF- κ B complex. Indeed, consistent with several prior studies of the effects of mitogens and cytokines on the activation of NF- κ B (13, 19, 38, 79), our current data suggest that phosphorylation of I κ B α triggers proteolytic degradation of this inhibitor. Specifically, phosphorylated I κ B α present in the Tax-expressing HTLV-I-infected T cells exhibits a remarkably shorter intracellular survival ($t_{1/2} < 5$ min) than the unmodified I κ B α present in the noninfected non-Tax-expressing Jurkat cells ($t_{1/2} > 2$ h) (Fig. 3B). While it is conceivable that Tax induces both specific kinases and proteases, which act independently to eliminate I κ B α , we suspect that these are contingent events and that phosphorylation of I κ B α serves as a molecular trigger for I κ B α degradation by one or more cellular proteases. Of note, our prior pulse-chase experiments have shown that the transiently expressed free I κ B α molecules are rapidly degraded ($t_{1/2} = 40$ min) even in unstimulated cells, albeit with slower kinetics than phosphorylated I κ B α ($t_{1/2} < 5$ min). However, when coexpressed with RelA, the intracellular survival of I κ B α is markedly prolonged (79). Thus, it seems likely that the sites involved in protease attack within I κ B α are masked when this inhibitor is complexed with RelA. In turn, phosphorylation may lead to the conformational changes in the I κ B α molecule that allows protease attack to begin even while the inhibitor is physically complexed with RelA. It also seems likely that constitutive and inducible pathways of I κ B α degradation exist.

In an attempt to identify inhibitors of the cellular protease mediating I κ B α degradation, we tested a number of different protease inhibitors, including TPCK, an agent which blocks chymotrypsin-like enzymes. However, while TPCK effectively blocked nuclear expression of NF- κ B and c-Rel, this agent also blocked the phosphorylation of I κ B α occurring in response to either Tax or TNF- α . Thus, TPCK appeared to act at a point prior to phosphorylation of the inhibitor rather than distally to block the protease(s) that degrades I κ B α . Similar inhibitory effects on I κ B α phosphorylation were obtained with pyrrolidinedithiocarbamate, a chelator and scavenger of free radicals known to inhibit NF- κ B induction in response to many stimuli, including Tax and TNF- α . This possibility is reminiscent of recent studies with the hepatitis B virus x protein, which have revealed that this transactivator mimics tumor promoters and acts by stimulating a PKC-dependent signaling pathway (46). However, Tax activation of NF- κ B/Rel appears to involve a PKC-independent pathway since Tax-mediated I κ B α phosphorylation and degradation are not inhibited by various PKC inhibitors, including staurosporin (data not shown). Studies are now under way to define the intracellular signaling pathway involved in Tax action.

In Tax-expressing cells, both RelA and c-Rel are constitutively expressed in the nucleus (reference 3 and Fig. 2). While the nuclear expression of RelA is likely the direct result of I κ B α phosphorylation and degradation, Tax induction of nuclear c-Rel expression appears to involve a more complex mechanism. As noted previously, our prior studies have demonstrated distinctly different kinetics of NF- κ B and c-Rel expression following PMA or TNF- α stimulation (26, 54). Nuclear NF- κ B expression occurs within minutes, while nuclear c-Rel expression is delayed for several hours after

stimulation and importantly requires *de novo* protein synthesis. Our current studies now demonstrate that NF- κ B activates *c-rel* gene expression via a κ B enhancer element located in the promoter-proximal region of the gene. In contrast, subsequent accumulation of nuclear c-Rel protein serves to inhibit RelA-mediated activation of the *c-rel* gene. These findings reveal an intriguing and unexpected regulatory network whereby different Rel proteins control not only their own expression but also that of other Rel family members and their cytoplasmic inhibitors (9, 25, 71, 79). Tax expression in HTLV-I-infected T cells may serve to override this autoregulatory cycle, leading to sustained high-level nuclear expression of both RelA and c-Rel. As such, this abnormal pattern of nuclear expression of these Rel-related proteins may contribute to HTLV-I-mediated T-cell transformation.

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