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Tax protein of human T-cell leukemia virus type I binds to the ankyrin motifs of inhibitory factor κB and induces nuclear translocation of transcription factor NF-κB proteins for transcriptional activation

(trans-activation/transcription/protein–protein interaction)

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ABSTRACT **Human T-cell leukemia virus type I causes adult T-cell leukemia and tropical spastic paraparesis, and its regulator protein Tax has been implicated in the pathogenic activity of human T-cell leukemia virus type I. Tax activates transcription of viral and cellular genes through specific enhancers: the 21-bp enhancer of human T-cell leukemia virus type I, the nuclear factor κB (NF-κB)-binding site of the interleukin 2 receptor α gene, and the serum-responsive element of c-*fos*. Tax binds to enhancer-binding proteins including cAMP-responsive element-binding protein, cAMP-responsive element modulator, transcription factor NF-κB p50 and $p67^{SRF}$, and associates with each enhancer DNA indirectly. In addition to this mechanism, we report here that Tax binds to inhibitory factor κBγ (I-κB) γ, which forms a complex with NF-κB protein heterodimer p50–p65 or homodimer p50–p50 and retains them in the cytoplasm. Tax binding to I-κBγ induces nuclear translocation of NF-κB p65. In association with this nuclear translocation of p65, transcription directed by the κB enhancer is strongly activated. Tax binds to the ankyrin motifs of I-κBγ, suggesting its possible interaction with many other proteins carrying ankyrin motifs contributing to various regulatory processes. This is a different mechanism of transcriptional activation by the oncoprotein Tax and seems to be independent from the trans-activation through indirect binding to enhancer DNAs.**

Human T-cell leukemia virus type I (HTLV-I) (1, 2) is the causative agent of adult T-cell leukemia (3–5) and tropical spastic paraparesis (6, 7). Its regulatory protein Tax is a transcriptional activator of viral and host cellular genes. Its target genes, which include interleukin 2, interleukin 2 receptor α (IL-2Rα), c-*fos*, c-*jun*, granulocyte–macrophage colony-stimulating factor, and parathyroid hormone-related protein, have suggested its involvement in the pathogenic activity of HTLV-I. In fact, Tax shows properties like a transforming protein, inducing immortalization of human T cells with $CD4^+$ phenotype (8), transformation of rodent fibroblasts (9), and tumorigenesis in transgenic mice carrying *tax* gene (10).

Tax activates transcription of viral and cellular genes through distinct enhancers—namely, the 21-bp enhancer of HTLV-I (11), the nuclear factor κB (NF-κB)-binding site of the IL-2Rα gene (12, 13), and the serum-responsive element of c-fos (14). These enhancers have no sequence homology to each other, and furthermore, Tax itself does not bind to these enhancer DNAs directly. However, Tax was recently found to bind to enhancer-binding proteins and thus binds to enhancer DNA indirectly; these DNA-binding proteins are cAMP-responsive element-binding protein and cAMP responsive element modulator for the 21-bp enhancer (15, 16), NF-κB p50 for the NF-κB-binding site (17), and $p67^{SRF}$ for serum-responsive element (17, 18). Moreover, Tax binding to these enhancer-binding proteins seems to play critical roles in trans-activation of transcription, as suggested by a series of Tax mutants (17).

In addition to the binding to these DNA-binding proteins, we reported (19) that Tax also binds to NF-κB p105, a precursor of NF-κB p50, and its complex formation was well correlated with the activation capacities of the Tax mutants on the κB site (19). NF-κB p105 itself and the Tax-p105 complex, however, did not bind to the κB DNA sequence. These findings strongly suggested another mechanism of trans-activation in addition to the indirect binding of Tax to enhancer DNA.

The κB p105 is a precursor of NF-κB p50 (20–22), and its C-terminal half has similar domains to those in the inhibitory factor κB (I-κB) family (23). The I-κB proteins form complexes with the active form of transcription factors NF-κB p50, p65, or c-Rel (24–27) and sequester the NF-κB proteins in the cytoplasm (28, 29), keeping them in the latent forms. These complexes have been proposed to be dissociated when cells are stimulated with certain signals for proliferation or differentiation, and the released NF-κB proteins are translocated into the nucleus to activate transcription (30, 31). Therefore, it is possible that Tax may interact with the ankyrin motifs in I-κB and NF-κB p105 and dissociate or interfere with the complexes of I-κB/NF-κB. In this paper, characterization of Tax binding to the I-κB protein and its effects on transcriptional activation are described.

MATERIALS AND METHODS

Cells and Plasmids. COS-7 and 293T kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% and 5% fetal calf serum, respectively.

For expression of proteins, two vectors were used: the pCG vector, which has a cytomegalovirus promoter and multicloning site (32) or its derivative pCG-HA vector, which carries the sequence for the influenza virus hemagglutinin (HA) epitope (33). The DNA fragments used for expression were as follows: for expression of I-κBγ, a fragment containing the entire coding and 3′-noncoding sequences was excised by *Hin*dIII from the mouse I-κBγ cDNA clone in Bluescript SK^- (23); for expression of the ankyrin motifs, the fragment (Ank-C) containing the sequence from the *Nco* I site (1485) to the termination code of the NF-κB p105 cDNA (22),

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Abbreviations: HTLV-I, human T-cell leukemia virus type I; IL-2Rα, interleukin-2-receptor α chain; NF-κB, nuclear factor κB; I-κB, inhibitory factor κB; GST, glutathione *S*-transferase; HA, hemagglutinin; LUC, luciferase; mAb, monoclonal antibody.

the fragment (Ank-P) from the *Xba* I site (1685) to *Pst* I (2633), or the fragment (Ank-B) from the *Xba* I site (1685) to *Bgl* II (2198); for expression of NF-κB p65, the cDNA sequence of p65 from the initiation codon to the termination codon amplified by PCR from the p65 cDNA clone (34). The expression plasmids of wild-type and mutant Tax were also all derivatives of pCG vector (19). An expression plasmid for glutathione *S*-transferase (GST) I-κBγ was constructed inserting the entire coding sequence of I-κBγ into a vector carrying GST-coding sequence.

***In Vitro* Formation of Protein Complexes.** Fusion proteins, GST-I-κBγ and His–Tax, were produced in *Escherichia coli*, as described (16, 19). The GST–I-κBγ (≈4 μg) in *E. coli* extract was purified by adsorbing onto glutathione-conjugated Sepharose beads and then incubated with purified His–Tax (16) under described conditions (19). After incubation for 8 hr, the beads were isolated, washed, and analyzed by immunoblotting with anti-Tax antibodies.

Transfection and Subcellular Fractionation. COS-7 and 293T cells were transfected with plasmids by the standard calcium phosphate precipitation procedure (19). After 2-day incubation, the cells were lysed in hypotonic buffer containing 0.5% Nonidet P-40, and the nuclear and cytoplasmic fractions were separated by centrifugation at 800 × *g* for 10 min. The nuclei precipitated were extracted with buffer containing 0.4 M NaCl, as described (19). These nuclear and cytoplasmic fractions were used directly for immunoprecipitation or immunoblot analysis.

Immunoprecipitation, Immunoblotting, and Immunostaining. Immunoprecipitation was done with either rabbit antiserum (Ab-9983) against the C-terminal region of NF-κB p105, the epitope of which is also contained in I-κBγ, or monoclonal antibody (mAb) 12CA5 to the HA epitope of influenza virus. The cell extracts were diluted 5-fold with NETG buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Nonidet P-40/1 mM EDTA/0.25% gelatin/0.02% sodium azide) and treated with one of the antibodies, and the immunocomplexes were collected on protein A-Sepharose CL4B (Pharmacia). The precipitates were separated in SDS/10% polyacrylamide gel, followed by immunoblotting, as described (19). The filter was incubated with anti-Tax antibody (35), and the bands were visualized with protein A–horseradish peroxidase conjugate and the enhanced chemiluminescence detection system (Amersham). For immunostaining, cells were fixed with acetone/methanol, 3:7, treated with mAb 12CA5 for 1.5 hr at 37°C and then incubated with fluorescein isothiocyanate-conjugated antimouse IgG for 1 hr at 37°C.

RESULTS

Tax Binds to I-κBγ. To examine interaction of the Tax protein with the I-κB family, we used mouse I-κBγ (23), which interacts with NF-κB p50, p65, and c-Rel. cDNA of I-κBγ was linked to the sequence of the HA epitope of the influenza virus genome to express HA-fused I-κBγ. HA–I-κBγ and Tax were coexpressed in 293T cells, an adenovirus-transformed human embryonic kidney cell line carrying simian virus 40 antigen. To demonstrate complex formation of Tax with I-κBγ, the cytoplasmic extract was treated with an antibody (Ab-9983) that recognizes the C terminus of I-κBγ. On immunoblot analysis of immunoprecipitates, Tax was detected when Tax and HA–I-κBγ were coexpressed (Fig. 1*A*, lane 4). Without transfection of HA–I-κBγ, Tax was not coprecipitated (lane 2), indicating that the endogenous level of I-κBγ was too low to precipitate a detectable amount of Tax. A mAb to the HA epitope gave almost identical results (Fig. 1*B*). These observations clearly indicate that Tax complexed with exogenously expressed I-κBγ in the cytoplasm.

To determine the functional significance of this complex, mutants of Tax were analyzed for their ability to complex.

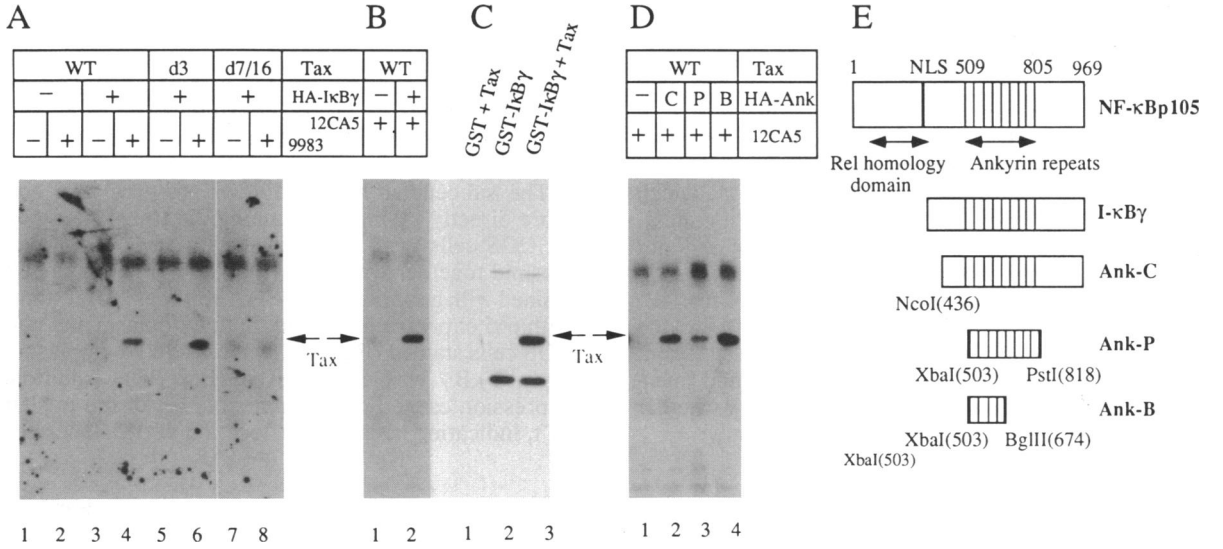

FIG. 1. Formation of a complex of Tax protein with I-κBγ and its binding site on I-κBγ. Interaction of Tax protein and I-κBγ or its fragments was analyzed by coimmunoprecipitation. (*A*) Derivatives of pCGTax expressing the wild-type (WT) Tax protein (lanes 1–4), d3 Tax mutant (lanes 5, 6), and d7/16 Tax mutant (lanes 7, 8) were cotransfected into 293T cells with a cDNA for mouse I-κBγ linked to the HA epitope of influenza virus (lanes 3–8) or with vector without an insert (lanes 1, 2). Cytoplasmic extracts were treated with normal rabbit serum (lanes 1, 3, 5, 7) or antiserum to the C-terminal region of I-κBγ (lanes 2, 4, 6, 8). The precipitates were subjected to immunoblotting for detection of Tax protein. The position of Tax protein is indicated by an arrow. (*B*) The same extracts as in lanes 2 and 4 were treated with mAb 12CA5 and analyzed similarly to those in *A*. (*C*) Direct binding of Tax to GST–I-κBγ. GST domain alone (lane 1) or GST–I-κBγ (lanes 2 and 3) was adsorbed to glutathione-Sepharose, and the isolated Sepharose beads were incubated with bacterially produced His–Tax (lanes 1 and 3), as described. Protein complexes on the Sepharose beads were isolated from the incubation mixture and analyzed by immunoblotting with anti-Tax antibodies. (*D*) Tax binding to the ankyrin motifs of I-κBγ. cDNA for HA–Ank-C (lane 2), HA–Ank-P (lane 3), or HA–Ank-B (lane 4) was cotransfected into 293T cells with pCGTax and cytoplasmic fractions were prepared (lane 1, cells lacking Ank plasmid). Extracts were treated with mAb 12CA5, and precipitates were analyzed by immunoblotting with anti-Tax antibody. (*E*) The sequence of mouse I-κBγ is almost identical (93% homologous) to the C-terminal domain of NF-κB p105 (22, 23); thus deletion mutants of I-κBγ, Ank-C, Ank-P, and Ank-B were constructed from the cDNA of p105. Ank-B contained four copies of the ankyrin motifs. NSL, nuclear localization signal.

The d3 mutant of Tax, which has a deletion at the N terminus, is fully active in trans-activation of the NF-κB enhancer, although it is inactive in activation of the 21-bp enhancer (19). The d3 mutant protein was coimmunoprecipitated with I-κBγ by antibody Ab-9983 against I-κBγ (Fig. 1*A*, lane 6) when I-κBγ was coexpressed, but without transfection of the I-κBγ expression plasmid, no d3 mutant protein was coprecipitated (data not shown). However, another Tax mutant, d7/16, which is inactive in the trans-activation of the κB site (19), did not form a complex (lane 8). These results strongly suggest that formation of a complex of Tax with I-κBγ correlates with the functional activities of Tax and thus should be significant in trans-activation of the NF-κB enhancer.

Direct binding of Tax to I-κBγ was tested with purified proteins: a GST fusion protein of I-κBγ was produced in bacteria, purified, and tested for binding to Tax protein *in vitro*. Purified His–Tax protein, a Tax protein fused to the 6-histidine sequence at the N terminus (16), was adsorbed onto a glutathione column in the presence of the GST-fusion protein of I-κBγ but was not adsorbed with GST alone (Fig. 1*C*). In these analyses with immunoblotting, two other bands were also detected by anti-Tax; however, these bands reflected bacterial proteins contaminated in the preparation of GST–I-κBγ because these bands were detected in the absence of His–Tax protein. These findings indicate that Tax protein binds directly to the I-κBγ protein but does not bind through cytoplasmic NF-κB p50, which complexes with I-κBγ and also with Tax protein (17).

Tax Binds to Ankyrin Motifs. I-κBγ contains eight repeats of ankyrin motifs, which is common to the I-κB family and is thought to interact with NF-κB proteins, such as p50, p65, and c-Rel. Therefore, we addressed the question of whether the ankyrin repeats of I-κBγ are responsive to Tax binding. Because I-κBγ consists of the C-terminal half of NF-κB p105, we used the C-terminal domain of p105 for preparation of I-κBγ fragments. The C-terminal domain of p105 (Ank-C) containing eight repeats of the ankyrin motif was further truncated into Ank-P and -B, which contained eight and four repeats of the ankyrin motif, respectively (see Fig. 1*E*), and these preparations were linked to the HA epitope at their N termini, constructing HA–Ank-C, HA–Ank-P, and HA–Ank-B. Each construct was coexpressed with Tax, and the cytoplasmic fraction was treated with mAb 12CA5, which recognizes the HA epitope. In all three cases, the immunoprecipitates contained Tax protein (Fig. 1*D*) but did not contain Tax protein in the absence of these fragments, clearly indicating that Tax complexed with HA–Ank-C, -P, or -B. Therefore, the ankyrin motifs were concluded to be the Tax-binding site, and four repeats of the motif were sufficient for its binding. Because I-κBα and Bcl-3 have five and seven ankyrin repeats, respectively, it is interesting to know whether or not Tax binds to other members of the I-κB family.

Tax Induces Nuclear Translocation of p65. I-κBγ binds to NF-κB p50, p65, and c-Rel through their ankyrin motifs and sequesters them in the cytoplasm (23). I-κB-α and -β (30) have also been shown to bind to some of these NF-κB proteins, but these complexes are dissociated by specific signals for cell growth or differentiation, allowing translocation of NF-κB proteins into the nucleus. As described above, I-κBγ also binds to Tax at the ankyrin motifs. Thus, we suspected that Tax may interfere with the binding of NF-κB p50, p65, or c-Rel to the ankyrin motifs of I-κBγ. We therefore examined the effects of Tax on the function of I-κBγ, which sequesters NF−κB proteins in the cytoplasm. An expression plasmid of p65 was transfected into 293T cells with or without I-κBγ, the cells were fractionated into cytoplasmic and nuclear fractions, and each fraction was subjected to immunoblotting for p65 detection. With the p65 expression plasmid, p65 was detected in both cytoplasmic and nuclear fractions (Fig. 2, lanes 1 and 2), although endogenous p65 was not detected with this assay (data not shown). Tax expression induced nuclear translocation of almost all p65 (lanes 3 and 4). Several bands of smaller molecular sizes were postulated to be degradation products of p65. Addition of protease inhibitors to the extracts resulted in increase of the intensity of the p65 band (data not shown), although the inhibitors did not significantly diminish those of the smaller bands, suggesting that p65 in these extracts was unstable after disruption of cells.

Coexpression of I-κBγ with p65 induced cytoplasmic accumulation of p65 and drastically reduced the nuclear level of p65 (lanes 5 and 6). This is consistent with the binding of p65 to cytoplasmic I-κBγ. When Tax was coexpressed in this system, almost all of the p65 was detected in the nuclear fraction (lanes 7 and 8), although most of the I-κBγ stayed in the cytoplasmic fraction (Fig. 2*B*). These results indicate that Tax suppressed the binding of I-κBγ to p65, resulting in the nuclear translocation of p65. Almost identical results were obtained with two mutants of Tax, d3 and d320, which are active in trans-activation of the κB enhancer (lanes 9–12), but were not with mutant d7/16, which is inactive in trans-activation (lanes 13 and 14). In studies on c-Rel expression, we have observed very similar nuclear localization to that described above for p65 (unpublished results).

The subcellular localization of p65 was also confirmed more directly by immunostaining the transfected cells (Fig. 3). COS cells were transfected with an HA-p65 expression plasmid together with I-κBγ and/or Tax, and the cells were stained with antibodies to the HA epitopes. After transfection with p65 expression plasmid, both cytoplasm and nucleus of COS cells stained (Fig. 3*A*), whereas only cytoplasm stained when I-κBγ was coexpressed (Fig. 3*B*). Additional Tax expression caused preferential staining of the nucleus (Fig. 3*C*), indicating nuclear translocation of p65. Therefore, Tax

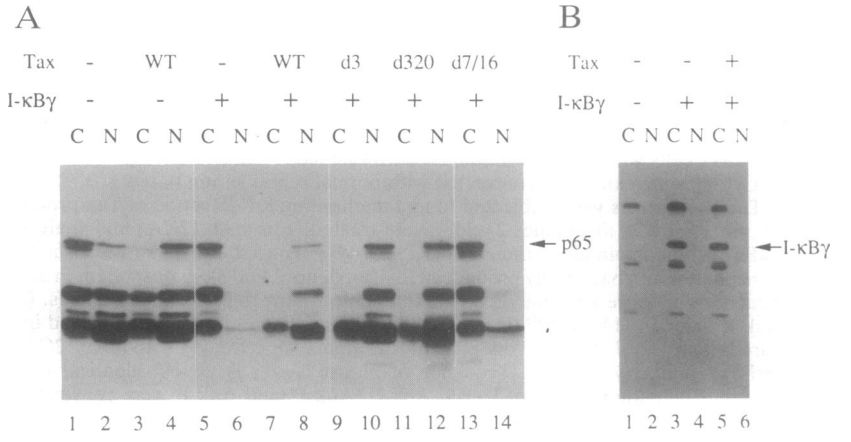

FIG. 2. (*A*) Tax-dependent nuclear localization of NF-κB p65. 293T cells were transfected with the p65 expression vector in the absence of Tax (lanes 1, 2), in the presence of Tax alone (lanes 3, 4), in the presence of I-κBγ alone (lanes 5, 6), or in the presence of both I-κBγ and wild-type (WT) Tax (lane 7, 8), the d3 Tax mutant (lanes 9, 10), the d320 Tax mutant (lanes 11, 12), or the d7/16 Tax mutant (lanes 13, 14). Cytoplasmic (C) and nuclear (N) fractions were analyzed directly by immunoblotting with antibodies to p65. (*B*) Tax-independent localization of I-κBγ in the cytoplasm. Cells were transfected with vector DNA without insert (lanes 1, 2), with the I-κBγ expression plasmid in the absence (lanes 3, 4) or the presence of Tax (lanes 5, 6). Cytoplasmic and nuclear fractions were examined by immunoblotting with anti-I-κBγ (Ab-9983).

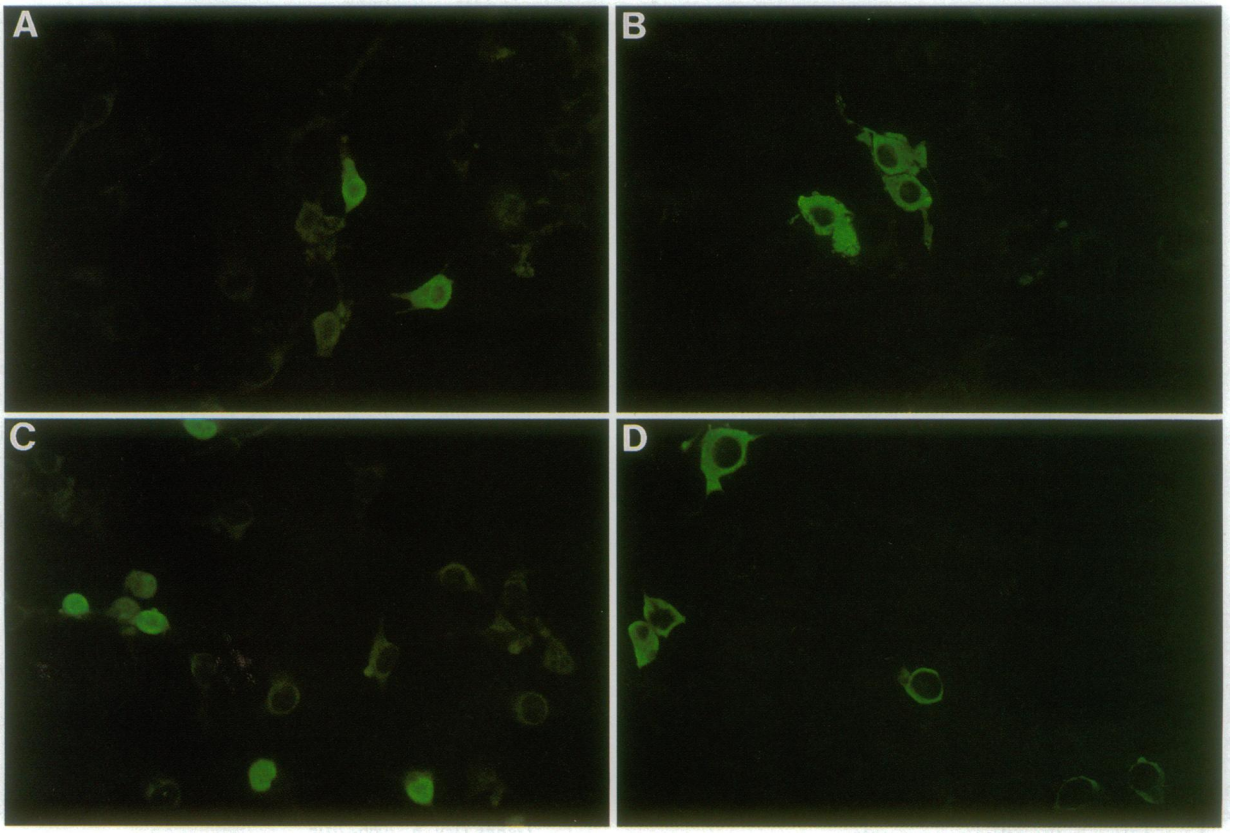

FIG. 3. Identification of subcellular localization of p65 by immunostaining. Cells transfected with HA-p65 expression plasmid with or without I-κBγ and/or Tax were stained by the indirect immunofluorescence method. Cells with p65 (*A*), p65 plus I-κBγ (*B*), p65 plus I-κBγ plus wild-type Tax (*C*), or p65 plus I-κBγ plus d7/16 mutant Tax (*D*) are shown.

apparently suppressed I-κBγ activity. However, expression of Tax mutant d7/16 did not induce nuclear translocation of p65 in the presence of I-κBγ (Fig. 3*D*). All these observations on *in situ* staining of the cells confirmed the results obtained by subcellular fractionation described above.

Trans-activation Correlates with Nuclear Translocation of p65. To demonstrate the effect of nuclear translocation of p65 on transcription, we examined gene expression that linked to the NF-κB binding site. A reporter plasmid, κB-LUC, containing a luciferase (LUC) gene under control of the NF-κB binding sequence of the IL-2Rα gene, was transfected into 293T cells. Expression of p65 enhanced the LUC expression 324-fold (Fig. 4, hatched bar 2); however, coexpression of I-κBγ almost completely suppressed this enhanced activity to the background level (hatched bar 3). This suppression is consistent with the cytoplasmic retardation of p65. An increased level of p65 restored the LUC activity, in part (hatched bar 4), suggesting the importance of a balance of p65 and I-κBγ for regulation of κB activity. Tax expression in this system induced 148-fold enhancement of LUC activity (hatched bar 5). Because the total level of p65 was not significantly affected by Tax expression (see Fig. 2), these observations clearly indicate that Tax protein counteracted the effects of I-κBγ, probably through binding to I-κBγ, and resulted in nuclear accumulation of p65. On the other hand, a combination of Tax and I-κBγ without p65 showed only 26-fold activation (hatched bar 6). Therefore, for efficient activation by Tax, the p65 sequestered in the cytoplasm is involved. Tax alone caused 117-fold activation in the absence of I-κBγ suppression (hatched bar 7), probably through nuclear translocation of endogenous p65 and its related proteins, and this activity was also reduced 26-fold when I-κBγ was coexpressed (hatched bar 6), suggesting that endogenous p65 is also regulated by I-κB and Tax.

These patterns of activation and suppression of LUC expression that are controlled by the combination of p65, Tax, and I-κBγ correlated well with the localization of p65 in the nucleus and cytoplasm, respectively. Thus, we conclude that Tax binding to the ankyrin motifs of I-κBγ either inhibits formation of the I-κBγ/p65 complex, dissociates the preformed complexes, or both and induces nuclear translocation of p65, which ultimately activates transcription.

DISCUSSION

In this study, we show that Tax binds to the I-κBγ protein through the ankyrin motifs and induces nuclear translocation of NF-κB p65, ultimately activating transcription through the κB enhancer. The ankyrin motifs of I-κBγ are the site of complex formation with NF-κB proteins (36), and the Tax binding is thus expected to compete with formation of I-κBγ/NF-κB-protein complexes, which retards the NF-κB proteins in the cytoplasm. Tax, therefore, may induce the nuclear translocation of NF-κB p65 through interference with the complex formation of I-κBγ with p65 and/or dissociation of the complex in the cytoplasm. These mechanisms can explain the previous observations that Tax increases the nuclear levels of active NF-κB p50 and p65 (37). In response to signals for cell proliferation or differentiation, the I-κB component in the I-κB/NF-κB complexes is phosphorylated, and the complexes are dissociated, resulting in translocation of the active NF-κB proteins into the nucleus for activation of specific gene expression (30, 31). Alternatively, signals induce a rapid degradation of I-κBα, thus leaving free forms of NF-κB proteins (38). Thus, direct binding of Tax to I-κBγ may bypass cellular regulatory processes triggered by phosphorylation or proteolysis.

This induction of protein translocation is a different mechanism of transcriptional activation induced by Tax protein

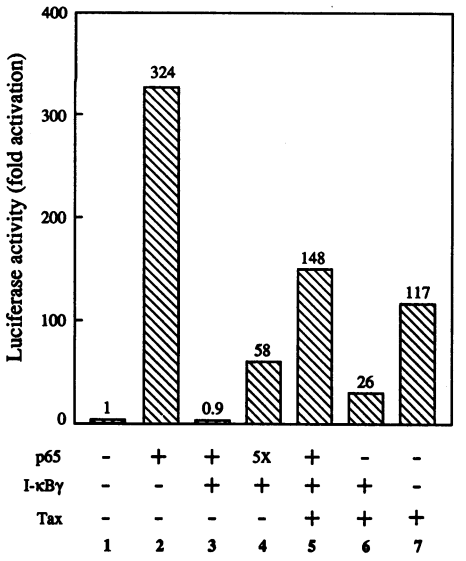

FIG. 4. Effect of Tax and I-κBγ on p65-dependent LUC expression. 293 T cells were transfected with combinations of the expression plasmids of p65 (0.1 μg), I-κBγ (1 μg), and Tax (0.02 μg), indicated by + together with a reporter plasmid containing the LUC gene fused to five repeats of the NF-κB site of the IL-2Rα gene and an enhancerless promoter of HTLV-I. Increase of LUC activity in cell extracts is shown relative to that after mock transfection, which was 2580 units/μg of protein per min. Activity depended on incubation time and protein concentration used. Mean values for three independent assays are presented. 5x, five times more DNA (0.5 μg) of p65 plasmid than in the other experiments.

from the other mechanism in which Tax binds to enhancer DNA through enhancer-binding proteins. In the latter mechanism, Tax binds to nuclear proteins cAMP-responsive element-binding protein and cAMP-responsive element modulator, which bind to the 21-bp enhancer (15, 16), NF-κB p50, which binds to the NF-κB enhancer (17), and $p67^{SRF}$, which binds to the CArG box enhancer (18). Therefore, we propose here that Tax can activate specific transcription through two mechanisms, translocation of active transcription factor(s) into the nucleus and binding to enhancer-binding proteins on the enhancer DNA.

Tax was first reported to be a nuclear protein (35) and, in fact, binds to nuclear factors as mentioned above (15, 16). However, the present study indicated that Tax is also present in the cytoplasm and regulates NF-κB transcription factors. Although the Tax protein analyzed here was in transfected cells, we had reported (35) that a fraction of Tax protein is present in the cytoplasm of HTLV-I-infected cells. Therefore, the present finding that Tax binds to I-κBγ in the cytoplasm indicates a physiological significance of cytoplasmic Tax protein in infected cells.

Tax binds to the ankyrin motifs in I-κBγ and NF-κB p105. This binding site suggests that Tax may bind to other factors carrying ankyrin motifs and may modulate protein–protein interactions through ankyrin repeats (39). Thus, it would be interesting to examine its abilities to bind to other members of the I-κB family, such as I-κBα and I-κBβ and various other proteins carrying ankyrin motifs.

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