IKKγ Mediates the Interaction of Cellular IκB Kinases with the Tax Transforming Protein of Human T Cell Leukemia Virus Type 1*

(Received for publication, March 2, 1999, and in revised form, April 7, 1999)

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The Tax oncoprotein of human T cell leukemia virus type 1 constitutively activates transcription factor NF-κB by a mechanism involving Tax-induced phosphorylation of IκBα, a labile cytoplasmic inhibitor of NF-κB. To trigger this signaling cascade, Tax associates stably with and persistently activates a cellular IκB kinase (IKK) containing both catalytic (IKKα and IKKβ) and noncatalytic (IKKγ) subunits. We now demonstrate that IKKγ enables Tax to dock with the IKKβ catalytic subunit, resulting in chronic IκB kinase activation. Mutations in either IKKγ or Tax that prevent formation of these higher order Tax-IKK complexes also interfere with the ability of Tax to induce IKKβ catalytic function in vivo. Deletion mapping studies indicate that amino acids 1–100 of IKKγ are required for this Tax targeting function. Together, these findings identify IKKγ as an adaptor protein that directs the stable formation of pathologic Tax-IKK complexes in virally infected T cells.

Studies with Tax-transgenic mice suggest that this viral/host interaction is required to maintain the transformed phenotype of HTLV-1-infected cells (5).

In quiescent T cells, the activity of NF-κB is controlled from the cytoplasmic compartment by virtue of its signal-dependent interaction with inhibitors, including IκBα (6). Recent studies have identified two cytokine-inducible IκB kinases (IKKs), termed IKKα and IKKβ, that target IκBα for degradation via phosphorylation at Ser-32 and Ser-36 (7). These two kinases form heterodimers and function as catalytic subunits within a 700–900-kDa multicomponent complex (8). Whereas IKKα and IKKβ are activated transiently in cells treated with the cytokine tumor necrosis factor-α (TNF-α) (8–10), Tax induces their constitutive expression in HTLV-1-infected T cells (11, 12). We have recently found that Tax-induced activation of both IKK and NF-κB requires the formation of Tax-IKK complexes (12). However, the precise mechanism of Tax action on IKKs remains unclear.

Here we provide several lines of experimental evidence indicating that Tax-directed IKK activation is mediated by IKKγ (also called NEMO, IKKAP1, or FIP-3), a recently identified subunit of TNF-responsive IKKs whose precise signaling function is unknown (13–16). First, interference with IKKγ expression in T cell transfectants inhibits Tax-mediated activation of NF-κB. Second, IKKγ and Tax interact stably in the context of a high molecular mass IκB kinase derived from HTLV-1-infected T cells. Third, overexpression of IKKγ in vivo is sufficient to target Tax specifically to ectopic IKKγ, whereas deletion of the N-terminal region of IKKγ eliminates this targeting function. The finding that IKKγ enables Tax to dock with cellular IκB kinases highlights an important missing link in the mechanism by which this oncoprotein activates the constitutive expression of NF-κB in HTLV-1-infected T cells.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit antisera specific for IKKγ and Tax (amino acids 321–353) have been described (13, 17). Anti-IKKα (H-744) and IKKβ (H-470) antibodies were obtained from Santa Cruz, Inc. Agarose beads conjugated to monoclonal anti-FLAG and anti-Myc antibodies were purchased from IBI-Kodak and Santa Cruz, respectively. Expression vectors for antisense IKKγ RNA (AS-IKKγ) (14), Tax (17), FLAG epitope-tagged IKKγ (18), and IKKγ (13) have been described. Deletion mutants of IKKγ were constructed by polymerase chain reaction using specific oligonucleotide primers (sequences available upon request) and subcloned into pcdNA3.1/Myc-His (Invitrogen). Chloramphenicol acetyltransferase (CAT) reporter plasmids contained either two tandem κB enhancers (κB-TATA-CAT) (18) or the HTLV1 5′ long terminal repeat (HTLV1 LTR-CAT) (19).

Cell Culture, Transfections, and CAT Assays—Jurkat T cells, RIP-deficient Jurkat T cells (20), HTLV-1-infected T cells (21, 22), and S107 plasmacytoma cells (23) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mm l-glutamine, and antibiotics. Jurkat and S107 cells were transfected via electroporation (24). Human 293T cells (25) were transfected using calcium phosphate precipitation (26). All CAT assays were performed as described (24).

Subcellular Fractionation and Biochemical Analyses—Cytoplasmic extracts were prepared by detergent lysis (27) in the presence of phosphatase and protease inhibitors (12). For gel filtration (9), cytosolic proteins (10 mg) were equilibrated in EBL buffer (24) and subjected to chromatography on a precalibrated Superose 6 column (Amersham Pharmacia Biotech). Unless indicated otherwise, immunoprecipitations were performed as described (12). Resultant immunocomplexes were fractionated by SDS-polyacrylamide gel electrophoresis and probed on polyvinylidene difluoride membranes using an enhanced chemiluminescence system (SuperSignal, Pierce). IκB kinase activity was measured during an adaptive immune response, antigen-stimulated CD4+ T lymphocytes become committed to an activation program that triggers a transient phase of clonal expansion (1). In contrast, infection with human T cell leukemia virus type 1 (HTLV-1) can lead to the loss of cell cycle control and development of an aggressive malignancy called adult T cell leukemia (2). The Tax oncoprotein encoded by HTLV-1 stimulates the constitutive nuclear expression of transcription factor NF-κB, which regulates antigen-directed T cell proliferation (3, 4).

* This work was supported by National Institutes of Health Grant RO1 AI33839 (to D. W. B.), by NCI, National Institutes of Health Training Grant T32 CA09385 (to Z.-L. C.), and by the Howard Hughes Medical Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: HTLV-1, human T cell leukemia virus type 1; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; IKK, IκB kinase; TNF, tumor necrosis factor-α.
**RESULTS AND DISCUSSION**

**Constitutive Activation of NF-κB by Tax Involves IKKγ but Not RIP**—In prior studies, we established that Tax binds to and persistently activates a TNF-responsive IκB kinase containing two catalytic subunits termed IKKα and IKKβ (12). To determine how Tax interacts with cellular IKKs, we first examined whether the Tax/IKK signaling axis involves the death domain kinase RIP, which is essential for TNF-induced activation of NF-κB (20, 28). For these studies, Jurkat human T cells containing a RIP null mutation (20) were cotransfected with a Tax expression vector (Tax-WT) and a CAT reporter plasmid containing two κB enhancers (κB-TATA-CAT). Parallel experiments were conducted with expression vectors containing point mutations that selectively disrupt the ability of Tax to access either the CREB/ATF (Tax-M47) or the NF-κB/Rel (Tax-M22) transcription factor pathway (17). As shown in Fig. 1A, Tax-WT potently stimulated NF-κB-directed transcription in both parental and RIP-deficient Jurkat T cells. Similar results were obtained with Tax-M47 but not Tax-M22, consistent with their differing capacities to activate TNF-responsive IKKs (12). These in vivo functional data clearly show that RIP is dispensable for Tax-induced activation of NF-κB.

RIP interacts specifically with IKKγ, an integral subunit of TNF-responsive IKKs (13–16). In this regard, Yamaoka et al. (13) have reported experiments with IKKγ-deficient rat fibroblasts, suggesting a requirement for this subunit in coupling Tax to NF-κB, whereas others (29) have identified an IKKγ-deficient pre-B cell line that is fully responsive to Tax. To determine whether IKKγ couples Tax to NF-κB in a more physiologically relevant setting, Jurkat T cells were cotransfected with Tax-WT, κB-TATA-CAT, and graded amounts of a vector that directs the synthesis of antisense IKKγ RNA (AS-IKKγ). As shown in Fig. 1B, AS-IKKγ inhibited Tax-induced transcription directed from the NF-κB-responsive reporter in a dose-dependent fashion. In contrast, interference with IKKγ protein expression failed to affect Tax-induced transcription from the HTLV-1 5′ long terminal repeat, which is activated by an NF-κB-independent mechanism (30). These functional studies demonstrate that IKKγ is required for the induction of NF-κB by Tax in the context of human T lymphocytes, the in vivo target for HTLV-1.

**Tax Interacts Stably with TNF-responsive IKKs Containing IKKγ**—IKKγ assembles with TNF-responsive IKKs primarily via its interaction with IKKβ (13–15). This catalytic subunit also associates with Tax in HTLV-1-infected T cells (12). To determine whether IKKγ is a core component of Tax-responsive IKKs, we first performed in vitro kinase assays using cytoplasmic extracts from SLB-1 and CS166 T cells. Whereas SLB-1 cells produce replication-competent virions (21), CS166 cells harbor a defective provirus that selectively expresses Tax (22). In these biochemical experiments, Tax, IKKα, IKKβ, and IKKγ were isolated by immunoprecipitation and assayed for IKK catalytic activity using a GST-IκBα fusion protein as substrate. As shown in Fig. 2A (lanes 2–4, top panel), GST-IκBα phosphorylating activity was readily detected in Tax and IKKα/IKKβ immunoprecipitates derived from SLB-1 cells. A significant amount of IKK activity was also detected in IKKγ immunoprecipitates (lane 5). Similar results were obtained with CS166 cells (Fig. 2A, bottom panel). This subunit compositional analysis establishes that IKKγ is associated with a constitutively active IκB kinase in both HTLV-1-infected and Tax-expressing T cells.

The TNF-responsive form of IKK that contains IKKγ corresponds to a 700–900-kDa multisubunit complex (13, 14). To explore the size distribution of Tax-associated IKKs, cytosolic proteins from HTLV-1-infected SLB-1 cells were fractionated by gel filtration and the resultant eluates were subjected to immunoprecipitation with anti-IκB antibodies. Consistent
with the size of TNF-responsive IKKs, the majority of constitutively active IKKs associated with Tax in SLB-1 cells were detected in fractions corresponding to a molecular mass exceeding 700 kDa (Fig. 2B). To determine whether IKKγ was also present in these Tax-IKK complexes, SLB-1 fractions containing peak kinase activity were subjected to immunoprecipitation with IKKγ-specific antibodies and assayed for the presence of either IKK activity or Tax protein. As shown in Fig. 2C, IkB kinase activity and Tax were readily detected in these IKKγ immunoprecipitates (lane 3, top and bottom panels). These biochemical data indicate that Tax associates with IKKγ in the context of a high molecular mass IkB kinase in HTLV-1-infected T cells.

To address the stability of these higher order Tax-IKKγ complexes, IKKγ was immunopurified from SLB-1 fractions containing peak kinase activity (>700 kDa) and washed at high stringency with escalating concentrations of NaCl and urea. We then monitored the dissociation of IKKα-IKKβ and Tax from these IKKγ immunocomplexes using in vitro kinase and immunoblotting assays, respectively. As shown in Fig. 2D (bottom panel), high concentrations of either dissociation agent failed to release Tax from IKKγ. Interactions between IKKγ and the IKKα-IKKβ catalytic subunits were also highly resistant to release, as inferred from our ability to detect significant levels of IKKγ-associated IkB kinase activity under identical washing conditions (Fig. 2D, top panel). These results confirm that Tax, IKKγ, and the catalytic subunits of IKK interact with high affinity, further underscoring the specificity and pathologic relevance of this viral/host interaction in HTLV-1-infected T cells.

IKKγ Mediates the Functional Interaction between Tax and IKKβ—Tax activates TNF-responsive IKKs primarily via its stimulatory effects on IKKβ (11), which interacts directly with IKKγ (13–15). To determine whether IKKγ directs the assembly of Tax-IKK complexes, mammalian 293T cells were transfected with expression vectors for FLAG-tagged IKKβ, IKKγ, and Tax. Cytoplasmic extracts were then prepared and subjected to immunoprecipitation with either monoclonal anti-FLAG antibodies (Fig. 3A, top and middle panels) or Tax-specific antibodies (Fig. 3A, bottom panel). When IKKβ immunocomplexes were probed on immunoblots for the presence of Tax, we found that IKKβ interacted weakly with Tax-WT, Tax-M22, and Tax-M47 in IKKγ-deficient cells (Fig. 3A, lanes 2, 5, and 8, top panel). In contrast, significant amounts of both Tax-WT and Tax-M47 were associated with IKKβ in cells coexpressing IKKγ (lanes 3 and 9). Under identical transfection conditions, Tax-M22 failed to interact appreciably with IKKβ in the presence of ectopic IKKγ (lane 6). This divergent result with Tax-M22 could not be attributed to inefficient ectopic expression, because comparable amounts of IKKγ and Tax protein were detected in each triple transfection (Fig. 3A, lanes 3, 6, and 9, middle and bottom panels). Coupled with our prior observation that Tax-M22 is defective for binding to endogenous IKKs (12), these data strongly suggest that IKKγ confers IKKβ targeting specificity to Tax.

To explore the functional consequences of these higher order interactions, expression vectors for IKKβ, IKKγ, and Tax were introduced into S107 plasmacytoma cell line. Importantly, S107 cells harbor a genetic defect that impairs NF-κB expression (23), thus providing a cellular background with minimal IkB kinase activity. Following transfection, ectopic IKKβ was immunopurified from S107 cytoplasmic extracts and monitored for catalytic activity using an in vitro kinase assay. As shown in Fig. 3B, all three of the Tax constructs failed to stimulate IKKβ kinase activity in the absence of ectopic IKKγ (lanes 2–4), whereas overexpression of IKKγ in cells harboring wild type Tax and Tax-M47 potently induced IKKβ (lanes 6 and 8). In contrast, Tax-M22 was unable to activate IKKβ in the presence of IKKγ (lane 7), consistent with its defect in endogenous IKK binding (12). These functional data correlate precisely with our biochemical results demonstrating that IKKγ directs the formation of Tax-M47-IKKβ but not Tax-M22-IKKβ complexes in mammalian 293T cell transfectants (Fig. 2B).

The N Terminus of IKKγ Is Required for Tax Targeting to IKKβ—Primary sequence analyses indicate that IKKγ contains a C-terminal leucine zipper domain, a central coiled-coil domain, and an N-terminal domain with no apparent secondary structural features (13–16). To define the sequences in IKKγ that mediate its Tax adaptor function, Tax and FLAG-tagged IKKβ were transiently expressed in 293T cells along with a panel of Myc epitope-tagged deletion mutants of IKKγ (Fig. 4A). Cytoplasmic extracts were prepared from transfected cells and subjected to immunoprecipitation with antibodies specific for each ectopic protein. The resultant immunocomplexes were then probed for the presence of Tax and IKKγ protein on immunoblots. As shown in Fig. 4B, removal of C-terminal sequences either abutting or encompassing the leucine zipper domain of IKKγ (mutants D1 and D2) had no detectable effect on the formation of IKKβ-Tax and IKKβ-IKK complexes (lanes 4 and 5, top and middle panels). However, deletion of the N-terminal region of IKKγ (amino acids 1–100, mutant D3) completely disrupted both of these interactions (lanes 6, top and middle panels). We consider these results to be significant, because the three IKKγ deletion mutants were comparably coexpressed with Tax in the cytoplasmic compartment (Fig. 4B, lower panels). Furthermore, parallel experiments conducted with Tax-M22 confirmed the specificity of these higher order interactions (Fig. 4B, lanes 7–12).
To extend these findings with ectopically expressed IKK\(\beta\), we monitored for either IKK activity (3). However, coexpression of Tax with IKK\(\gamma\) led to potent activation (lane 3). Consistent with their ability to target Tax to ectopic IKK\(\beta\), IKK\(\beta\) mutants D1 and D2 were fully competent to mediate Tax activation of endogenous IKK catalytic activity (lanes 4 and 5). In contrast, the N-terminal deletion mutant of IKK\(\gamma\) failed to reconstitute a functional Tax-IKK signaling axis (lane 6). We conclude that the N terminus of IKK\(\gamma\) is required for stable integration of this subunit into endogenous IKK, which in turn renders the holoenzyme susceptible to persistent activation by Tax.

In summary, we have found that the IKK\(\gamma\) subunit of TNF-responsive IKKs is an essential core component of Tax-associated IKKs in HTLV-1-infected T cells. Higher order complexes containing Tax, IKK\(\gamma\), and the IKK\(\alpha\)-IKK\(\beta\) catalytic subunits are highly resistant to dissociation in vitro, underscoring the specificity of this pathologic viral/host interaction. In vivo reconstitution experiments demonstrate that IKK\(\gamma\) directs the assembly of Tax-IKK\(\gamma\) complexes, resulting in the persistent expression of IkB kinase activity. Thus, IKK\(\gamma\) functions in Tax-mediated IKK activation at the level of Tax-IKK\(\gamma\) docking. By analogy, this targeting mechanism may reflect an important role for IKK\(\gamma\) in coupling TNF-responsive IKKs to upstream physiologic activators, such as NIK and MEKK1 (31).

Acknowledgments—We thank Shoji Yamaoka, Alain Israel, Frank Mercurio, David Rothwarf, Michael Karin, and Brian Seed for reagents.

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