Domain-specific Interaction with the IκB Kinase (IKK) Regulatory Subunit IKKγ Is an Essential Step in Tax-mediated Activation of IKK*

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The human T-cell leukemia virus type 1 Tax oncoprotein deregulates the NF-κB signaling pathway by persistently stimulating a key signal transducer, the IκB kinase (IKK). Tax physically associates with the IKK regulatory subunit, IKKγ, although the underlying biochemical mechanism and functional significance remain unclear. We show that the Tax-IKKγ interaction requires two homologous leucine zipper domains located within IKKγ. These leucine zipper domains are unique for the presence of a conserved upstream region that is essential for Tax binding. Site-directed mutagenesis analysis revealed that a leucine-repeat region of Tax is important for IKKγ binding. Interestingly, all the Tax mutants defective in IKKγ binding failed to engage the IKK complex or stimulate IKK activity, and these functional defects can be rescued by fusing the Tax mutants to IKKγ. These results provide mechanistic insights into how Tax specifically targets and functionally activates the cellular kinase IKK.

Human T-cell leukemia virus type 1 (HTLV-I)† is an onco- genetic retrovirus etiologically associated with the development of an acute T-cell malignancy termed adult T-cell leukemia (1, 2). The HTLV-I genome encodes a transactivator protein, Tax, which plays a pivotal role in the induction of T-cell transformation (3, 4). Tax not only regulates viral gene expression directed from the long terminal repeat (LTR) of HTLV-I (5–7) but also transactivates a large array of cellular genes, an action contributing to the initiation of cellular transformation (8). Activation of viral gene expression by Tax involves its physical interaction with CREB/ATF, a family of basic leucine zipper (bZIP)-containing transcription factors (9–11). Tax interacts with the bZIP DNA binding domain of CREB/ATF proteins, promoting their binding to the Tax-responsive enhancer located in the HTLV-I LTR (12–14). Additionally, Tax also recruits transcriptional coactivators to the HTLV-I LTR (15–18). Induction of many cellular genes by Tax is mediated through activation of NF-κB (19), a key regulator of genes involved in cell activation and growth (20, 21).

Unlike CREB/ATF, which is constitutively expressed in the nucleus, NF-κB is normally sequestered in the cytoplasmic compartment by physical interaction with inhibitors, including IκBo and related proteins (22, 23). NF-κB nuclear expression can be transiently induced upon cellular stimulation by a variety of agents, such as T-cell mitogens and proinflammatory cytokines (21, 24, 25). These agents stimulate the activity of a multisubunit IκB kinase (IKK), which phosphorylates IκBo, targeting this inhibitor for degradation through the ubiquitin-proteasome pathway (26). In Tax-expressing cells, IKK is chronically activated (Refs. 27–29, reviewed in Ref. 30), which is associated with constitutive IκBo phosphorylation and NF-κB nuclear expression (31–33).

The IKK complex is composed of two catalytic subunits, IKKα and IKKβ (34), and a regulatory subunit, IKKγ (also named NEMO, IKKAP1, or FIP-3) (35–38). Although purified recombinant IKKα and IKKβ can directly phosphorylate IκBo in vitro (37, 39–41), formation of the signal-responsive IKK holoenzyme in vivo requires IKKγ (35, 36, 42, 43). IKKγ lacks a kinase domain but contains long stretches of coiled-coil protein interaction sequences, including a C-terminal leucine zipper (LZ) motif (36). Precisely how IKKγ participates in IKK signaling is unknown, but this regulatory protein has been proposed to interact with upstream activators in response to cellular activation signals (36). In this regard, IKK can be activated by several upstream kinases, including MEK kinase 1 (MEKK1), NF-κB inducing kinase (NIK), and certain isoforms of protein kinase C (27, 39, 44–48). However, the physiological role of these upstream kinases in IKK regulation in vivo has not been demonstrated.

Given the persistent nature of Tax-induced IKK activation, this viral specific pathway may be mediated by a specific mechanism. Interestingly, Tax is stably associated with the IKK complex in HTLV-I infected T cells (49). These findings raise the possibility that constitutive association of Tax with the IKK complex may be responsible for persistent IKK activation in Tax-expressing cells. More recently, several independent studies have demonstrated that the IKKγ subunit of IKK physically interacts with Tax and promotes the association of Tax with the IKK catalytic subunits (50–52). However, how Tax recognizes IKKγ and whether the Tax-IKKγ interaction serves as an essential step in Tax-mediated IKK activation is not currently known. In this paper, we present data demonstrating that the Tax-IKKγ interaction requires two homologous LZs present in IKKγ and a leucine-rich region present in Tax. This molecular

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§ The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; LTR, long terminal repeat; bZIP, basic leucine zipper; LZ, leucine zipper; IKK, IκB kinase; UR, upstream region; LR, leucine repeats; LRR, leucine-repeat region; IP, immunoprecipitation; luc, luciferase; HA, hemagglutinin; GST, glutathione S-transferase; CC, coiled coil; IB, immunoblotting; KA, immunocomplex kinase assay; CREB, cAMP response element-binding protein.
interaction is essential for Tax-mediated stimulation of IKK and subsequent activation of NF-κB.

MATERIALS AND METHODS

Plasmid Constructs and Antibodies—The cDNA of IKKα, IKKβ, and IKKγ have been described previously (54). IKKγ truncation mutants were generated by site-directed mutagenesis (Stratagene) using the wild-type HA-tagged murine IKKγ as template. The IKKγΔLRc mutant harbors a deletion of the four leucine repeats of LZc (amino acids 315–336); IKKγΔLRn has a deletion of the first leucine repeat of LZn (amino acids 101–115); ΔURc and ΔURn have deletions of the UR of LZc and LZn, respectively. Tax mutants harboring amino acid substitutions were generated with the same strategy using wild-type Tax expression vector as template (see Fig. 1C). Expression vectors were constructed by inserting the IKKγ cDNA upstream of wild-type or mutant forms of Tax. The anti-HA- and anti-IKKγ monoclonal antibodies were from Roche Molecular Biochemicals and Imgenex Corporation, respectively. The anti-Tax monoclonal antibody was from Milipore.

Immunoprecipitation (IP) and Immunoblotting Assays—Human 293 kidney carcinoma cells were seeded in 0.1% gelatin-treated 6-well plates (1 × 106 cells/well) and transfected using DEAE-dextran with the indicated cDNA expression vectors. The DNA amounts used for the transfections were normalized based on the expression efficiency of each of the expression vectors: 100 ng for IKKα and IKKβ, 50 ng for IKKγ, 250 ng for Tax mutants, 0.5 μg for Tax and its mutants. After 40 h, the recipient cells were lysed in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1:100 (v/v) of a protease inhibitor mixture (54)). Whole cell lysates were subjected to IP in the radioimmunoprecipitation buffer as described previously (54), and the precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting (54). For direct immunoblotting analyses of proteins, cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting.

Luciferase Reporter Gene Assays—Jurkat T cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% of antibiotics. The Jurkat cell line expresses Tax constitutively lacking expression of IKKγ, which was generated by somatic mutagenesis (42). The parental and mutant Jurkat cells (5 × 106) were transfected using DEAE-dextran (55) with 200 ng of luciferase reporter plasmids together with the indicated effector cDNA expression vectors. At 48-h post-transfection, the recipient cells were subjected to extraction using a reporter lysis buffer (luciferase reagent, Promega) at about 100 μl/106 cells. Luciferase activity was detected by mixing 5 μl of extract with 25 μl of luciferase substrate (Promega) and measured with a luminometer. For reporter gene assays performed with 293 cells, the cells were seeded in 24-well plates (2.5 × 105 cells/well) and transfected using DEAE-dextran with 20 ng of cDNA-luciferase reporter plasmids together with the indicated effector cDNA expression vectors. After 40 h, cells were lysed in a lysis buffer containing 20 mM Hepes (pH 7.6), 250 mM NaCl, 0.5% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM EDTA, 20 mM p-nitrophenylphosphate, 0.1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a previously described protease inhibitor mixture (56). HA-IKKα and HA-IKKβ were isolated by IP using anti-HA antibody. Immune complexes were washed three times with lysis buffer, once with lysis buffer supplemented with 1 × urea, and twice with a kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 1 mM EDTA, 2 mM p-nitrophenylphosphate, and 2 mM dithiothreitol). Kinase assays were performed as described (57) using GST-IκBα-(1-54) as substrate. IKK proteins in the immune complexes were detected by immunoblotting.
Fig. 1. Two homologous LZ motifs of IKKγ contribute to its physical interaction with Tax. A, schematic representation of murine IKKγ and its truncation mutants. The five coiled coils, predicted according to Lupas et al. (60) (Window 14), are depicted by boxes (CC1-CC5) with the amino acid positions shown above. Two homologous leucine zipper domains LZn and LZc are indicated. The truncated forms of IKKγ are designated by the specific amino acid residues retained. B, interaction of Tax with IKKγ or IKKβ truncation mutants. 293 cells were transfected with the indicated IKKγ truncation mutants along with the Tax expression vector. The full-length (FL) IKKγ, IKKγ(151–412), and IKKγ(1–312) were tagged with HA epitope, whereas the IKKγ(47–412) and IKKγ(69–412) were tagged with the Myc epitope. Cell lysates were subjected to IP using anti-HA followed by IB using anti-Tax and anti-Myc (lanes 1 and 3). The full-length (FL) Tax, Tax(1–151), Tax(151–412), and Tax(125–412) were also tagged with HA, whereas the Tax(35–151) and Tax(60–412) were tagged with the Myc epitope. Cell lysates were subjected to IP using anti-HA followed by IB using anti-Tax (upper). The expression level of the transfected IKKγ and Tax proteins were analyzed by anti-HA plus anti-Myc (middle) and anti-Tax (lower), respectively. The Tax-LZn mutants were described in detail under “Materials and Methods.” C, sequence alignment of the two homologous LZ domains present in murine (mIKKγ) and human (hIKKγ) IKKγ. Identical and similar amino acid residues are shown in bold. The conserved upstream region (UR) and leucine-repeet region (LR) are indicated below the figure, and the four conserved leucines in the leucine heptad repeats are underlined. D, interaction of Tax with IKKγ mutants harboring internal deletions. 293 cells were transfected with the indicated cDNA expression constructs. Cell lysates were subjected to IP with anti-HA followed by IB using anti-Tax (upper). The expression level of the IKKγ mutants and Tax were analyzed by IB using anti-HA (middle) and anti-Tax (lower), respectively. The multiple bands of IKKγ resulted from its constitutive phosphorylation, as they could be converted to a single band following in vitro phosphatase treatment (data not shown).

Secondary structure prediction using the PREDATOR program (61) revealed a short α-helix located in the UR of the Tax LRR (data not shown), which is homologous to the UR of IKKγ (Fig. 2A, UR). A similar motif is also present in the Tax protein encoded by HTLV-II (Fig. 2A, Tax2). We examined the role of the Tax LRR in IKKγ binding by introducing amino acid substitutions (Fig. 2B) followed by examining the ability of the Tax mutants to bind IKKγ (Fig. 2C). Wild-type Tax strongly interacted with IKKγ (Fig. 2C, upper, lane 2), whereas the M22 mutant failed to mediate this interaction (lane 3). Remarkably, single substitution of each of the conserved leucines (Leu-124, Leu-131, Leu-138) in the LRR of Tax completely prevented Tax from binding IKKγ (Fig. 2C, upper, lanes 4–6). Mutation of asparagine 117 to glycine (N117G) and arginine 116 to serine (R116S) also resulted in a significant inhibitory effect (Fig. 2C, lanes 9 and 12). On the other hand, mutation of two amino acids located downstream of the LRR (Thr-145 and Val-152) only moderately affected the Tax-IKKγ interaction (Fig. 2C, lanes 7 and 8). Single substitution of the conserved amino acids present in the UR of Tax each caused a partial inhibition of Tax-IKKγ interaction (Fig. 2C, lanes 13 and 14). Double and quadruple substitutions of these conserved amino acids completely disrupted the IKKγ binding activity of Tax (Fig. 2C, lanes 10 and 11). Parallel immunoblot analyses revealed that all the Tax mutants, as well as the cotransfected IKKγ, were stably expressed in the cells (Fig. 2C, middle and lower panels). These results indicate that the LRR of Tax is important for Tax binding to IKKγ. Of course, it is unlikely that the LRR is the only region of Tax required for Tax-IKKγ interaction, because some Tax variants mutated outside of this region are also defective in NF-κB activation (59, 62) or IKKγ binding (51).

**Tax/IKKγ Interaction Is Required for Recruiting Tax to IKKα/IKKβ**—Prior studies have shown that Tax does not appecriably interact with the IKK catalytic subunits; however, this oncprotein forms stable complexes with both IKKα and IKKβ in the presence of IKKγ (50–52). To understand the mechanism by which IKKγ promotes the association of Tax with the IKK catalytic subunits, we determined whether this “docking” function of IKKγ required its physical interaction with Tax. For these studies, we examined the ability of the various IKKγ mutants to promote Tax association with IKKα or IKKβ by co-IP. As expected (50–52), Tax failed to associate with IKKβ when these two proteins were coexpressed in 293 cells (Fig. 3A, lane 2). However, the two proteins became stably associated when wild-type IKKγ was also expressed in the cells (lane 3). More importantly, the IKKγΔLRc, which retained significant Tax binding activity (see Fig. 1D, lane 3), was able to stimulate strong association between Tax and IKKβ (lane 4).

In contrast, consistent with their poor Tax binding activity, the IKKγΔULRc and IKKγΔLRn exhibited a weak docking activity (lanes 5 and 6). Moreover, the IKKγ mutant harboring a deletion of the ULR was completely unable to promote the Tax-IKKβ association (Fig. 3A, lane 7); this result was in agreement with the Tax-binding deficiency of this IKKγ mutant (see Fig. 1D, lane 6). We also examined the effect of these IKKγ mutants on Tax-IKKα association and obtained similar results (data not shown). Thus, the function of IKKγ in promoting the binding of Tax to IKKβ is correlated with its ability to physically interact with Tax.

To further assess the role of Tax/IKKγ interaction in IKKγ-mediated recruitment of Tax to the IKK catalytic subunits, we examined the function of various Tax mutants in associating with IKKα/IKKβ. Wild-type Tax formed a stable complex with IKKβ when these two proteins were coexpressed with IKKγ (Fig. 3B, lane 2). Similarly, two Tax mutants (T145A and V152A) competent in IKKγ binding also associated with IKKβ.
FIG. 2. A leucine-repeat region of Tax is important for IKKγ binding. A, sequence showing the heptad leucine repeats (LR) and the upstream region (UR) of the Tax proteins encoded by HTLV-I (Tax) and HTLV-II (Tax2). The amino acid residues conserved between the UR of Tax LRR and IKKγ L2 (shown in Fig. 1C) are bold. The leucines present in the heptad leucine repeats are underlined. B, amino acid substitutions within and downstream of the Tax LRR. M22 is a mutant previously generated by random mutations (59). The new Tax mutants were designated based on the positions of the mutated amino acids and the replacing residues (for example, Q107G harbors a substitution of glutamine 107 by glycine). C, interaction of IKKβ with Tax and Tax mutants. 293 cells were transfected with Tax or its mutants either alone (lane 1) or together with HA-tagged murine IKKγ (lanes 2–14). The cell lysates were subjected to IP using anti-HA followed by IB using anti-Tax (upper). The level of expression of HA-IKKγ and Tax mutants were analyzed by direct IB using anti-HA (middle) and anti-Tax (lower), respectively.

(Fig. 3B, lanes 7 and 8). In sharp contrast, the Tax mutants incapable of IKKγ binding failed to engage IKKβ (Fig. 3B, lanes 3–6 and 9–11). Parallel co-IP assays to detect the IKKβ-dependent Tax/IKKγ association revealed a similar result (data not shown). Together, these data suggest that the Tax-IKKγ interaction is required for recruiting Tax to the IKK catalytic subunits.

Tax Mutants Defective in IKKγ Binding Fail to Stimulate IKK Kinase Activity and NF-κB Transcriptional Function—We next determined the functional significance of Tax-IKKγ interaction in Tax activation of this cellular kinase and its target transcription factor NF-κB. Immunocomplex kinase assays were performed to analyze the ability of the various Tax mutants to stimulate IKK catalytic activity in 293 cells. In the absence of exogenously transfected IKKγ, Tax only weakly induced the transfected IKK catalytic subunits (IKKα and IKKβ) in 293 cells (data not shown, also see Fig. 5B, lane 2). This was probably caused by the relatively low level of endogenous IKKγ expressed in these cells (see Fig. 5D). However, together with transfected IKKγ, the wild-type Tax potently activated the IKK catalytic subunits (Fig. 4A, lane 2), as demonstrated by the phosphorylation of the substrate IκBα, (lower, IκBa-P) and IKKα/β autophosphorylation (upper, IKKαβ-P). More importantly, Tax M22 and the other Tax mutants defective in IKKγ binding were inactive in IKK activation (lanes 3–6 and 9–11). In contrast, those Tax mutants competent in IKKγ binding (T145A and V152A) significantly stimulated IKK catalytic activity (lanes 7 and 8). We extended these functional studies by performing kinase assays using Jurkat Tag cells. As previously reported (28), Tax was able to stimulate significant activity of IKK in these T cells in the absence of transfected IKKγ (Fig. 4B, upper, lane 2); this result was consistent with the presence of high amounts of endogenous IKKγ in Jurkat Tag cells (Fig. 5D, lane 5). More importantly, under these conditions, the function of Tax mutants in IKK activation was also well correlated with their ability to physically interact with IKKγ (lanes 3–11). We next performed reporter gene assays to further evaluate the function of Tax mutants in stimulating NF-κB signaling. Each of the Tax mutants were transfected into 293 cells (Fig. 4C) or Jurkat T cells (Fig. 4D) along with a luciferase reporter driven by the NF-κB target enhancer, κB (κB-luc). Wild-type Tax potently stimulated the κB enhancer, resulting in marked induction of luciferase activity (Fig. 4, C and D, bar 2). A significant κB stimulatory activity was also obtained with two Tax mutants (T145A and V152A) that were able to bind IKKγ (bars 7 and 8). However, none of the Tax mutants defective in IKKγ binding could activate the κB enhancer (bars 3–6 and 9–11). Thus, the Tax/IKKγ physical interaction is required for Tax-mediated stimulation of IKK and subsequent activation of NF-κB.

Fusion of Interaction-defective Tax Mutants to IKKγ Rescues Their Functional Defect in NF-κB Signaling—The observation that Tax mutants defective in IKKγ binding fail to activate IKK provides strong support for the idea that the Tax-IKKγ interaction plays an essential role in Tax activation of IKK. We reasoned that fusion of the interaction-deficient Tax mutants to IKKγ might rescue their functional defect in NF-κB signaling. To test this idea, we generated fusion proteins composed of IKKγ and Tax mutants and determined their function in stimulating IKK catalytic activity and NF-κB transcriptional func-
When linked to the C terminus of IKKγ, all the Tax mutants were efficiently recruited to IKKβ (Fig. 5A, lanes 5–11) and IKKα (data not shown). Immunocomplex kinase assays were performed to examine whether fusion of the Tax mutants with IKKγ restored their function in stimulating IKK catalytic activity. As expected, wild-type Tax potently stimulates IKK activity when coexpressed with IKKγ in 293 cells (Fig. 5B, lane 4), whereas M22 or the other Tax mutants defective in IKKγ-binding failed to activate IKK (Figs. 5B, lane 5 and 4A). Remarkably, when these interaction-deficient Tax mutants were fused to IKKγ, their IKK-stimulatory function was efficiently restored (lanes 6–12). A parallel kinase assay performed with Jurkat Tag cells revealed that the IKKγ-Tax chimeras significantly stimulated IKK activity in these T cells (Fig. 5C).

We further validated the finding presented above by examining the ability of the IKKγ-Tax chimeras to stimulate NF-κB transcription activity. We took advantage of an IKKγ-deficient Jurkat cell line, JM4.5.2, recently isolated in our laboratory (42). In these mutant T cells, Tax is unable to activate the κB enhancer unless exogenous IKKγ is provided (42). As expected, a marked luciferase activity was detected when IKKγ was coexpressed with the wild-type Tax (Fig. 6A, bar 3), but no reporter gene expression was detected when IKKγ was coexpressed with the Tax mutants defective in IKKγ binding (bars 4–10). More importantly, consistent with the kinase assays, fusion of these Tax mutants to IKKγ efficiently restored their NF-κB activation function (bars 11–17). Similar results were obtained in a parallel reporter gene assay performed with wild-type Jurkat cells (Fig. 6B). In these cells, Tax efficiently induced κB-Luc reporter when transfected at an adequate amount (0.4 μg, bar 4) and weakly induced the reporter at a low dose (80 ng, bar 3). In contrast, none of the Tax mutants tested was able to activate the κB-Luc when transfected at either low or high doses (Figs. 6B, bars 5–11 and 4D). However, all the IKKγ-Tax chimeras significantly induced the luciferase activity (bars 12–18). We found that the level of κB activation by the chimeras was lower in Jurkat cells than in the IKKγ-deficient JM4.5.2 cells (compare Fig. 6, A versus B). Although the amounts of DNA used for transfecting the wild-type Jurkat cells were lower, increasing the DNA amounts did not further enhance the level of reporter activation (data not shown). It is likely that the lower efficiency of NF-κB activation in wild-type Jurkat cells is caused by the association of IKKα/IKKβ with the endogenous IKKγ, which would interfere with binding of the IKK catalytic subunits to the transfected IKKγ-Tax chimeras. Nevertheless, a functional rescue, as a result of fusion between IKKγ and Tax mutants, was clearly detected in the wild-type Jurkat cells. Taken together, these results demonstrate that the IKKγ-Tax interaction serves as an essential step in Tax-triggered NF-κB signaling.

**DISCUSSION**

The multisubunit kinase IKK responds to a large variety of cellular stimuli as well as the signal triggered by the HTLV Tax protein (26, 30, 63). Although precisely how IKK responds to the diverse signals remains unclear, recent genetic evidence suggests that the regulatory subunit, IKKγ, plays an essential role in IKK activation by both cellular signals and Tax (35, 42, 43). An interesting structural feature of IKKγ is the presence of several stretches of coiled-coil motifs, including a C-terminal
LZ (36). This LZ has been proposed to mediate interaction with upstream IKK activators (34). In the present study, we have identified an N-terminal LZ (LZn), which shares striking overall sequence similarities with the C-terminal LZ (LZe in this study). A unique feature of the IKKγ LZs is the presence of a highly conserved UR motif. We have shown that the LZn is particularly important for IKKγ binding to Tax; deletion of either the heptad leucine repeats (LRn) or the URn severely crippled the Tax binding activity of IKKγ (Fig. 1D). The LZc seems to be also involved in the IKKγ-Tax interaction; deletion of URc significantly diminished the Tax binding activity of IKKγ, although deletion of the leucine-repeat region of this LZ (LRc) did not produce a significant effect.

Previous studies suggested that the region of Tax containing the M22 mutation is important for Tax dimerization and activation of NF-κB (64). Interestingly, this region contains three continuous heptad leucine repeats (Fig. 2A). Single substitution of the conserved leucines by alanines results in Tax mutants incapable of IKKγ binding, suggesting the importance of these hydrophobic residues in the Tax-IKKγ interaction. It is
Our data indicate that the UR of Tax may possibly form an terminus of Tax has been suggested to interact with CREB shown that several other Tax mutants harboring LRR muta-
tions involve many different mechanisms. In this regard, the pathways (19), because the development of these two pathways the LRR of Tax and is defective in IKK
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