The Tax Oncoprotein of Human T-cell Leukemia Virus Type 1 Associates with and Persistently Activates IκB Kinases Containing IKKα and IKKβ*

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The Tax oncoprotein of human T-cell leukemia virus type 1 (HTLV1) chronically activates transcription factor NF-κB by a mechanism involving degradation of IκBa, an NF-κB-associated cytoplasmic inhibitor. Tax-induced breakdown of IκBa requires phosphorylation of the inhibitor at Ser-32 and Ser-36, which is also a prerequisite for the transient activation of NF-κB in cytokine-treated T lymphocytes. However, it remained unclear how Tax interfaces with the cellular NF-κB/IκB signaling machinery to generate a chronic rather than a transient NF-κB response. We now demonstrate that Tax associates with cytokine-inducible IκB kinase (IKK) complexes containing catalytic subunits IKKα and IKKβ, which mediate phosphorylation of IκBa at Ser-32 and Ser-36. Unlike their transiently activated counterparts in cytokine-treated cells, Tax-associated forms of IKK are constitutively active in either Tax transfectants or HTLV1-infected T lymphocytes. Moreover, point mutations in Tax that ablate its IKK-binding function also prevent Tax-mediated activation of IKK and NF-κB. Together, these findings suggest that the persistent activation of NF-κB in HTLV1-infected T-cells is mediated by a direct Tax/IKK coupling mechanism.

The NF-κB/Rel family of transcription factors plays an important regulatory role in T-cell homeostasis and antigen-driven proliferation (1–4). In quiescent T lymphocytes, NF-κB is trapped in the cytoplasm by various inhibitory proteins, including IκBa (5). In response to mitogenic signals, IκBa is targeted for destruction by the ubiquitin-proteasome pathway and then NF-κB translocates to the nucleus (6). Proteolytic inactivation of IκBa requires signal-dependent phosphorylation of the inhibitor at Ser-32 and Ser-36 (7–10). Recent studies have identified two cytokine-inducible IκB kinases (IKKs)1, termed IKKα and IKKβ, which appear to form heterodimers and catalyze these site-specific modifications in the context of a multisubunit enzyme complex (11–15).

Human T-cell leukemia virus type 1 (HTLV1) is the etiologic agent of an aggressive malignancy of activated CD4+ T lymphocytes (16). The HTLV1 provirus encodes a 40-kDa oncoprotein, termed Tax, which potently induces the constitutive nuclear expression of NF-κB (17–19). Studies with Tax-transgenic mice suggest that this viral/host interaction is required to maintain the transformed phenotype of HTLV1-infected cells (20). We demonstrated recently that Tax converts IκBa into a labile proteasome substrate by a mechanism involving phosphorylation of the inhibitor at Ser-32 and Ser-36 (7, 21, 22). These findings suggested that Tax induces a chronic NF-κB response by acting upstream of one or more IKKs (7). However, the precise mechanism by which Tax accesses the host NF-κB signaling pathway remained unknown.

We demonstrate here that cytokine-inducible IκB kinases containing IKKα and IKKβ, which normally function in a transient manner (12–14), are expressed as constitutively active signal transducers in HTLV1-infected T lymphocytes. These activated forms of IKK associate stably with Tax when the oncoprotein is expressed in either transiently transfected or virus-infected cells. Point mutations in Tax that ablate its NF-κB-inducing activity also prevent the formation of functional IKK/Tax complexes. Our findings suggest that Tax persistently activates IKK proteins via a direct binding mechanism, resulting in the sustained degradation of IκBa and chronic nuclear expression of NF-κB in HTLV1-infected cells.

EXPERIMENTAL PROCEDURES

Reagents—The anti-IKKα antiserum was generated by immunizing rabbits with a synthetic peptide corresponding to amino acids 1–29 of human IKKα, which was covalently coupled to keyhole limpet hemocyanin.2 Purified polyclonal antibodies specific for IKKβ were provided by Frank Mercurio (Signal Pharmaceuticals, Inc., San Diego, CA). Polyclonal (23) and monoclonal (Tab-170) anti-Tax antibodies were obtained from Bryan Cullen (Duke University) and John Brady (NCI, Bethesda, MD), respectively. Complementary DNAs encoding wild-type and mutant forms of (23) were cloned into the expression vector pCMV4 (24). Recombinant glutathione S-transferase (GST)-IκBa fusion proteins were overexpressed and purified by affinity chromatography as described (12).

Cell Culture and Transfections—Jurkat T-cells and HTLV1-infected T-cell lines (25–27) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. Where indicated, Jurkat cells (5 × 10⁶ cells/ml) were stimulated for 10 min with human tumor necrosis factor-α (TNF) (40 ng/ml; Promega). Human 293T cells (28) were maintained in DMEM supplemented as described above and transfected using the calcium phosphate precipitation method (29).

Immunoprecipitation and Immunoblotting—Cytoplasmic extracts were prepared from cultured cells by detergent lysis (30) in the presence of phosphatase (1 mM Na₃VO₄, 1 mM NaF, 10 μM Na₃MoO₄, 1 mM Na₂VO₃) and protease (31) inhibitors. Lysates were adjusted to a final concentration of 30 mM HEPES (pH 7.0), 250 mM NaCl, and 5 mM EDTA before the addition of specific antisera. Immunoprecipitations were

1 The abbreviations used are: IKK, IκB kinase; HTLV1, human T-cell leukemia virus type 1; GST, glutathione S-transferase; TNF, tumor necrosis factor-α; PAGE, polyacrylamide gel electrophoresis.

2 J. Hawiger, R. A. Veach, X.-Y. Liu, and S. Timmons, unpublished results.
Kinase reactions (2019). We demonstrated previously that Tax stimulates phospho-
sient nuclear action of NF-
activate an IKK, resulting in the sustained rather than tran-
TNF (7–10). As such, we reasoned that Tax might persistently
the transient activation of NF-
22). Site-specific phosphorylation of IκB is also required for
response to the cytokine TFN (7–10). As such, we reasoned that Tax might persistently
resulting in the sustained rather than transient nuclear action of NF-κB. In this regard, recent studies have identified a multisubunit IKK complex containing two interactive catalytic components, termed IKKα and IKKβ, which mediate site-specific phosphorylation of IκB at Ser-32 and Ser-36 in TNF-treated cells (11–15). However, the relationship between these cytokine-inducible IKKs and Tax remained unknown.

To explore this relationship, cytoplasmic and nuclear extracts were prepared from three HTLV1-transformed T-cell lines, including MT-2, SLB-1, and C8166. Whereas the MT-2 and SLB-1 lines produce replication-competent virions (25, 26), C8166 cells harbor a defective provirus that selectively expresses Tax (27). Subcellular extracts were also prepared from Jurkat T-cells, which are TNF-responsive (32) and transformed by an HTLV1-independent mechanism. As demonstrated in gel shift assays, treatment of Jurkat T-cells with TNF led to the rapid induction of NF-κB DNA binding activity in the nuclear compartment (Fig. 1A, lanes 1 and 2, top panel). However, NF-κB was constitutively activated in each of the three HTLV1-infected lines (Fig. 1A, lanes 3–5, top panel). This constitutive pattern of nuclear NF-κB activity correlated with the expression of Tax protein in the cytoplasmic compartment of MT-2, SLB-1, and C8166 cells (Fig. 1A, lanes 3–5, bottom panel).

To monitor IKK activity in HTLV1-infected cells, in vitro phosphorylation assays were performed using a GST-IκBα (amino acids 1–54) fusion protein as substrate (12). Endoge-
nous IKKs were isolated by immunoprecipitation with either IKKα or IKKβ peptide-specific antisera and incubated with the GST-IκBα substrate and [γ-32P]ATP. Consistent with prior studies (11–15), IκB kinase activity directed by either IKKα or IKKβ in Jurkat T-cells was modestly stimulated by treatment with TNF for 10 min (Fig. 1B, lanes 1 and 2). However, in HTLV1-infected T-cells, IKKα- and IKKβ-associated kinase ac-
tivities were constitutively expressed (Fig. 1B, lanes 3–5). These IKK activities were specific for Ser-32 and Ser-36 of IκBα, because replacement of both sites with alanine in the GST-IκBα substrate eliminated phosphoryl group transfer (Fig. 1B, lanes 6–10). As demonstrated by immunoblotting (Fig. 1C), steady-state levels of the IKKα protein were reduced in HTLV1-infected cells relative to uninfected Jurkat T-cells (top panel), whereas IKKβ protein expression was modestly elevated (bottom panel). These studies suggest that Tax persistently activates endogenous IKKs by a post-translational mechanism. Because the predominant form of IKK appears to be dependent on the activities of both IKKα and IKKβ (13), we
IkK SDS-PAGE and probed on immunoblots with a mixture of C8166 cells. Tax immunocomplexes were then fractionated by SDS-PAGE, transferred to ImmunoBlot polyvinylidine difluoride membranes, and immunoblotted with a combination of IKKα- and IKKβ-specific antibodies. C, cytoplasmic extracts from SLB-1 cells were subjected to immunoprecipitation with normal immunoglobulin heavy chains (IgH) are indicated.

These results provide direct biochemical evidence that Tax associates stably with IKKs in HTLV1-infected cells.

Persistent Activation of IKK Proteins by Tax Requires the Formation of Tax-IKK Complexes—In light of these findings, we reasoned that the association of Tax with IKKs might be a prerequisite for IKK activation. To test this hypothesis, 293T cells were transfected with cDNA expression vectors for wild-type Tax (Tax-WT) and forms of the oncoprotein containing missense mutations that selectively disrupt its ability to access either the CREB/ATF (Tax-M47) or the NF-κB/Rel (Tax-M22) transcription factor pathway (23). Subcellular extracts were then prepared and subjected to gel shift, co-immunoprecipitation, and in vitro phosphorylation analyses. As shown in Fig. 3A (top panel), Tax-M22 failed to induce the nuclear expression of NF-κB DNA binding activity in transfected cells (lane 3), whereas wild-type Tax and the Tax-M47 mutant were fully competent to execute this function (lanes 2 and 4). All three of the Tax constructs were expressed at comparable levels in the cytosol of 293T transfectants (Fig. 3A, bottom panel).

To assess the status of endogenous IKK activity in these Tax-expressing cells, cytoplasmic extracts were subjected to immunoprecipitation with antibodies for either Tax, IKKα, or IKKβ and the resultant immune complexes were monitored for GST-IκBα phosphorylating activity. As shown in Fig. 3B (top panel), immune complexes containing either wild-type Tax or Tax-M47 possessed a significant level of IKK activity (lanes 2 and 4). In contrast, immunoprecipitates derived from cells transfected with either a blank expression vector or the Tax-M22 cDNA failed to affect GST-IκBα phosphorylation (lanes 1 and 3). Similar qualitative results were obtained in experiments conducted with IKKα (Fig. 3B, middle panel) and IKKβ (Fig. 3B, lower panel) immunoprecipitates, confirming that the mutation in Tax-M22 disrupted the functional interplay between Tax, IKK, and NF-κB.

To determine whether the mutation in Tax-M22 affected its

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**Fig. 2. Constitutively active IKKs associate stably with HTLV1 Tax.** A, cytoplasmic extracts derived from the indicated cell lines were subjected to immunoprecipitation with a Tax-specific antisera. Resultant immune complexes were assayed for either IκB kinase activity with the indicated substrates (upper panel) or probed for the presence of Tax protein on immunoblots with monoclonal antibodies (lower panel). B, cytoplasmic extracts from the indicated cell lines were subjected to immunoprecipitation with monoclonal anti-Tax antibodies. Resultant immune complexes were fractionated by SDS-PAGE, transferred to polyvinylidine difluoride membranes, and immunoblotted with a combination of IKKα- and IKKβ-specific antibodies. C, cytoplasmic extracts from SLB-1 cells were subjected to immunoprecipitation with normal serum (lane 1) or the indicated IKK-specific antibodies (lanes 2 and 3), and resultant immune complexes were probed with monoclonal anti-Tax antibodies on immunoblots as described in B.

Infer that both of these interactive catalytic subunits are affected in cells harboring HTLV1.

**HTLV1 Tax Associates Physically with Cytoplasmic IKKs—**To explore the possibility that Tax persistently activates IKKs via specific protein/protein interactions, Tax was immunoprecipitated from the cytosol of HTLV1-infected T-cells and assayed for the presence of IκBα kinase activity. As shown in Fig. 2A, GST-IκBα phosphorylating activity was detected in Tax immunoprecipitates isolated from virally infected lymphocyte lines (lanes 2–4), but not in control immunoprecipitates derived from Jurkat T-cells (lane 1). The IκBα kinase activity associated with Tax exhibited the appropriate sequence specificity, because disruption of Ser-32 and Ser-36 in IκBα resulted in loss of Tax-dependent phosphorylation (Fig. 2A, lanes 3 and 5–8). These results suggest that Tax forms stable cytoplasmic complexes with active IKKs in HTLV1-infected T-cells.

To determine whether either IKKα or IKKβ mediates this Tax-associated kinase activity, monoclonal anti-Tax antibodies were used to isolate cytoplasmic Tax from MT-2, SLB-1, and C8166 cells. Tax immunocomplexes were then fractionated by SDS-PAGE and probed on immunoblots with a mixture of IKKα and IKKβ peptide-specific antibodies. As shown in Fig. 2B (lanes 2–4), endogenous IKKα was detected in association with Tax in each of the HTLV1-infected cell lines tested, whereas a Tax-bound form of the IKKβ protein was also evident in SLB-1 cells. To extend these findings, we used peptide-specific antibodies to immunoprecipitate either IKKα or IKKβ from SLB-1 cells and then assayed for the presence of Tax in the resultant IKK immunocomplexes. Tax was readily detected in complexes containing either of these IKKs, albeit at higher levels in the IKKα immunoprecipitates (Fig. 2C, lanes 2 and 3).

**Fig. 3. Activation of IKK and NF-κB by HTLV1 Tax correlates with its IKK-binding function.** Nuclear and cytoplasmic extracts were prepared from 293T cells (1 × 10⁶) following transient transfection with the indicated expression vectors (7.5 μg each). A, nuclear extracts (5 μg) were added to reaction mixtures containing a [γ-32P]ATP and the GST-IκBα substrate. Reaction products were resolved by SDS-PAGE, and phosphoproteins were visualized by autoradiography (top panel). B, cytoplasmic extracts were subjected to immunoprecipitation with peptide-specific antisera for either Tax (top panel), IKKα (middle panel), or IKKβ (bottom panel). Immune complexes were assayed for IκB kinase activity in reaction mixtures containing [γ-32P]ATP and the GST-IκBα substrate. Reaction products were resolved by SDS-PAGE, and phosphoproteins were visualized by autoradiography. C, cytoplasmic extracts were subjected to immunoprecipitation with an IKKα-specific antisera. Resultant immune complexes were fractionated by SDS-PAGE and probed on immunoblots with antisera for either IKKα (top panel) or HTLV1 Tax (bottom panel). Positions of molecular size markers (in kilodaltons) and immunoglobulin heavy chains (IgH) are indicated.
IKK-binding function, cytoplasmic extracts from these transfectants were subjected to immunoprecipitation with an IKK-specific antisera and resultant immune complexes were analyzed for the presence of either IKKa or Tax. Comparable amounts of endogenous IKKa protein were immunoprecipitated from cells overexpressing either wild-type Tax, Tax-M22, or Tax-M47 (Fig. 3C, lanes 2–4, top panel). Consistent with their capacity to activate both IKK and NF-κB, wild-type Tax and Tax-M47 were detected in association with IKKa (Fig. 3C, lanes 2 and 4, bottom panel). However, formation of Tax/IKK complexes was not apparent in cells overexpressing Tax-M22, which fails to activate IKK and NF-κB (Fig. 3C, lane 3, bottom panel). Taken together, these findings provide a strong correlation between the IKK-binding function of Tax and the ability of this oncoprotein to persistently activate IKK and NF-κB.

**DISCUSSION**

The Tax protein of HTLV1 and the cytokine TNF both activate NF-κB by a mechanism involving signal-dependent phosphorylation of IκBα at Ser-32 and Ser-36 (7–10). In turn, hyperphosphorylated IκBα is targeted for destruction by the ubiquitin-proteasome pathway (21, 33). The IκBα gene is under negative-feedback control, which provides a negative-feedback loop to ensure the transient action of NF-κB. The IκB kinase (IKK) signal transducer are the NF-κB-inducing kinase (NIK) and the NIK-binding protein kinase kinase kinase (MEKK1), both of which have been shown to activate NF-κB and IKK proteins when overexpressed in vivo (36–39). Alternatively, Tax could function to displace an inhibitory subunit within the IKK complex. Indeed, indirect evidence for the existence of cellular IKK inhibitors has been reported previously (12, 40).

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