Persistent Activation of NF-κB by the Tax Transforming Protein Involves Chronic Phosphorylation of IκB Kinase Subunits IκKβ and IκKγ*

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The Tax transforming protein encoded by human T-cell leukemia virus type 1 (HTLV1) persistently activates transcription factor NF-κB and deregulates the expression of downstream genes that mediate cell cycle entry. We recently found that Tax binds to and chronically stimulates the catalytic function of IκB kinase (IKK), a cellular enzyme complex that phosphorylates and inactivates the IκB inhibitory subunit of NF-κB. We now demonstrate that the IκK catalytic subunit and IκKγ regulatory subunit of IKK are chronically phosphorylated in HTLV1-infected and Tax-transfected cells. Alanine substitutions at Ser-177 and Ser-181 in the T loop of IκKβ protect both of these IKK subunits from Tax-directed phosphorylation and prevent the induction of IκB kinase activity. Each of these inhibitory effects is recapitulated in Tax transfectants expressing the bacterial protein YopJ, a potent in vivo agonist of T loop phosphorylation. Moreover, ectopically expressed forms of IκKβ that contain glutamic acid substitutions at Ser-177 and Ser-181 have the capacity to phosphorylate a recombinant IκKγ substrate in vitro. We conclude that Tax-induced phosphorylation of IκKβ is required for IκKβ activation, phosphoryl group transfer to IκKγ, and acquisition of the deregulated IKK phenotype.

To initiate an adaptive immune response, T lymphocytes execute a signal transduction program that triggers the transient induction of transcription factor NF-κB, activation of downstream growth-related genes, and cell cycle entry (1). Part of this program is regulated from the cytoplasm by IκBa, an inhibitory subunit of NF-κB, and an inducible IκB kinase called IκK1 (2). In response to immune system cues such as the cytokine tumor necrosis factor-α (TNF), IKK phosphorylates IκBa at Ser-32 and Ser-36 (2). In turn, phosphorylated IκBa is degraded and NF-κB translocates to the nucleus (2).

The most well-characterized form of IKK contains two catalytic subunits, termed IκKa and IκKβ, and a regulatory subunit called IκKγ (NEMO) (2). In response to TNF, IκKβ is rapidly phosphorylated, activated, and down-regulated within 30 min (3). The relevant phosphoacceptors in IκKβ have been mapped to a region in its catalytic domain that shares strong homology with “T loop” regulatory sequences found in members of the mitogen-activated protein kinase kinase (MAP2K) family of enzymes (3). Consistent with this structural link, members of the MAP2K kinase (MAP3K) family of enzymes have been implicated in TNF-induced activation of IKK (4).

In contrast to their transient action in TNF-treated cells, IKK and NF-κB are constitutively activated in T lymphocytes infected with human T-cell leukemia virus type 1 (HTLV1) (5). This process is mediated by the Tax transforming protein of HTLV1 and appears to play an essential role in the pathogenesis of HTLV1-associated disease (5). Chronic stimulation of IKK catalytic activity by Tax is dependent on IκKγ, which directs the assembly of Tax-IKK complexes (5). Tax also binds to and activates MEKK1, a MAP3K that phosphorylates IκK in vitro (6). The dual specificity of Tax for these two enzymes may promote chronic phosphorylation of IKK and acquisition of the deregulated IKK phenotype. However, the phosphorylation status of IKK in Tax-expressing cells has not been examined.

We now demonstrate that IκKβ and IκKγ are chronically phosphorylated in Tax-expressing cells and HTLV1-infected T lymphocytes. Alanine replacements at Ser-177 and Ser-181 in the T loop of IκKβ inhibit Tax-directed phosphorylation of both subunits and block the chronic stimulatory effects of Tax on IκB kinase activity. Moreover, Tax-induced phosphorylation of IκKβ and IκKγ is antagonized by the bacterial protein YopJ, a potent in vivo inhibitor of T loop phosphorylation and IκKβ catalytic activity (7). The finding that Tax-induced phosphorylation of IκKγ is contingent upon IκB kinase activity may reflect IκKγ-mediated phosphorylation of IκKγ within individual Tax-IKK signaling complexes. Consistent with this finding, constitutively active forms of IκKβ have the capacity to phosphorylate a recombinant IκKγ substrate in vitro.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal anti-IκK antibodies (H-470, FL-419) and monoclonal anti-HA antibodies (F-7) were purchased from Santa Cruz, Inc. Monoclonal (M2) and polyclonal anti-FLAG antibodies were purchased from Sigma. Rabbit antisera specific for Tax have been described (8). Monoclonal anti-Tax antibodies (LT4) were kindly provided by Dr. Yuetsu Tanaka (Okinawa-Asia Research Center, Japan). Expression vectors for Tax, YopJ, and IKK have been described (8–12). HA-tagged IκKβ (13) was subcloned into pcCMV4 (14). The luciferase reporter plasmid NF-κB-Luc was obtained from Stratagene. The reporter plasmid HTLV1 LTR-Luc was engineered by subcloning the firefly luciferase gene into HTLV1 LTR-CAT (15).

Cell Culture, Transfections, and Reporter Assays—HeLa and 293T cells (16) were maintained in DMEM with 10% fetal bovine serum, 2 mM glutamine, and antibiotics. HeLa cells (1 x 10⁶) were transfected using Effectene (Qiagen), whereas 293T cells were transfected using calcium phosphate (17). Jurkat and MT-2 T cells were cultured in RPMI containing 55 µM mercaptoethanol and the supplements listed above.

mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MAP2K, mitogen-activated protein kinase kinase; PVDF, polyvinylidene difluoride; TNF, tumor necrosis factor-α.
Tax-induced Phosphorylation of IKK

RESULTS AND DISCUSSION

Tax-dependent Phosphorylation of IKK in Vitro—Prior studies have established that IKK directs the assembly of Tax/IKK complexes (5). However, the biochemical mechanism by which Tax stimulates IKK catalytic activity in these complexes remains unknown. To explore the role of phosphorylation in Tax/IKK signaling, HeLa cells were transfected with vectors for Tax, IKKα, and wild-type Tax. Parallel transfections were performed with mutants of Tax that are selectively defective for either CREB/ATF (Tax-M74) or NF-κB (Tax-M22) activation (8). We then isolated IKKβ complexes from recipient cell extracts by immunoprecipitation and subjected them to in vitro kinase assays in the presence of [γ-32P]ATP. As shown in Fig. 1A (top panel), the phosphorylation status of IKKβ was essentially unaffected by Tax in the absence of ectopic IKKγ (lanes 1 and 2). Programming cells with this Tax docking subunit resulted in significant phosphorylation of both IKKβ and IKKγ (lane 4). Similar results were obtained in experiments with Tax-M47 (lane 6), but not Tax-M22 (lane 5), consistent with their differing capacities to engage and activate IKK (18). This pattern of phosphorylation was not attributable to inefficient or variable protein expression (lower panels).

Phosphorylation of Ser-177 and Ser-181 in the T loop of IKKβ is required for its activation by TNP (3). To explore the role of Ser-177 and Ser-181 in Tax-dependent phosphorylation of IKKβ, HeLa cells were transfected with vectors for Tax, IKKγ, and either wild-type IKKβ or a mutant containing alanine replacements at these two sites (IKKβ-SA). We then prepared IKKβ (Fig. 1B) or Tax (Fig. 1C) immunoprecipitates for in vitro kinase assays. As shown in Fig. 1B, top panel, mutations affecting Ser-177 and Ser-181 in IKKβ completely blocked its phosphorylation in the presence of Tax (lanes 3 and 6). These mutations also prevented Tax-induced phosphorylation of IKKγ. Similar results were obtained with a kinase-dead mutant of IKKβ that is defective for ATP binding (IKKβ-KM, lanes 7–9) (10). These mutations had no significant effect on IKKβ protein levels (middle panel). However, we detected a significant shift in the electrophoretic mobility of IKKγ when coexpressed with Tax and wild-type IKKβ, consistent with a change in its phosphorylation status (bottom panel). All of these findings were recapitulated with Tax immunoprecipitates (Fig. 1C), indicating that the kinase activity responsible for IKKβ and IKKγ subunit phosphorylation is stably associated with Tax. We conclude that Ser-177 and/or Ser-181 in the T loop of IKKβ are required for Tax-directed phosphorylation of IKKβ and IKKγ in vitro.

Tax-dependent Phosphorylation of IKK in Vivo—We next used [32P]orthophosphate to metabolically label endogenous IKK in MT-2 cells. This transformed T lymphocyte line is chronically infected with HTLV1 and expresses high constitutive levels of IκB kinase activity (18). Parallel experiments were conducted with Jurkat T lymphocytes, which are transformed by an HTLV1-independent mechanism. Endogenous IKK was immunoprecipitated from the corresponding extracts, fractionated by SDS-PAGE, and analyzed by autoradiography. As shown in Fig. 2A, IKKδ and IKKγ were both hyperphosphorylated in MT-2 cells as compared with Jurkat cells (top panel, lanes 3 and 4). The observed pattern of IKK phosphorylation

![Fig. 1. Tax-dependent phosphorylation of IKK in vitro.](image-url)
could not be attributed to cell type-specific differences in the steady-state level of IKK protein expression (lower panels, lanes 3 and 4). These findings demonstrate that endogenous IKKβ and IKKγ are chronically phosphorylated in the physiologically relevant setting of HTLV1-infected T cells.

To extend these findings, HeLa cells were transfected with various combinations of Tax and IKK expression vectors and then metabolically labeled with [32P]orthophosphate. Ectopic IKKβ was isolated from cytoplasmic extracts using anti-FLAG M2-agarose beads and assayed for IκB kinase activity in vivo. These in vitro results correlated strongly with the in vivo phosphorylation data shown in Fig. 1B.

To explore the functional consequences of IKKβ phosphorylation, IKKβ complexes were immunopurified from HeLa cell transfectants expressing Tax, IKKγ, and either wild-type IKKβ or IKKγ. Ectopic IKKβ complexes were isolated from cytoplasmic extracts using anti-FLAG M2-agarose beads and assayed for IκB kinase activity in the presence of GST-IκBα (1 μg) and γ-32P]ATP. Phosphoproteins were resolved by SDS-PAGE and visualized by autoradiography (top panel). Relative levels of IKKβ protein were determined by immunoblotting with IκBα-specific antibodies (bottom panel).

**FIG. 2.** Tax induces phosphorylation of IKKβ and IKKγ in vivo. A, Jurkat and MT-2 T cells were radiolabeled with [32P]orthophosphate for 8 h. Cytoplasmic extracts (400 μg) were subjected to immunoprecipitation with the indicated monoclonal antibodies. Resultant complexes were fractionated by SDS-PAGE and analyzed for 32P incorporation (top panel). IKK protein content was determined by immunoblotting with subunit-specific antibodies (middle and lower panels). B, HeLa cells (1 × 10⁶) were transfected with vectors for Tax (100 ng), murine IKKγ (Myc epitope-tagged, 100 ng), and FLAG-tagged forms of either wild-type IKKβ (WT), IKKβ-SA, or IKKγ.SA. Cells were radiolabeled with [32P]orthophosphate for 8 h. Ectopic IKKγ complexes were isolated from cytoplasmic extracts using anti-FLAG M2-agarose beads and fractionated by SDS-PAGE. Resolved proteins were subjected to autoradiography (top panel) and immunoblotting with IKK subunit-specific antibodies (middle and bottom panels). C, HeLa cells were transfected as described in B. Ectopic IKKβ was isolated from cytoplasmic extracts with anti-FLAG M2-agarose beads and assayed for IκB kinase activity in the presence of GST-IκBα (1 μg) and γ-32P]ATP. Phosphoproteins were resolved by SDS-PAGE and visualized by autoradiography (top panel). Relative levels of IKKβ protein were determined by immunoblotting with IKKβ-specific antibodies (bottom panel).

**FIG. 3.** YopJ prevents Tax-induced activation of IKK and NF-κB. A, 293T cells (5 × 10⁵) were transfected with a Tax expression vector (1 μg), the indicated amounts of an effector plasmid encoding FLAG-tagged YopJ, and either the NF-κB-Luc or HTLV1 LTR-Luc reporter plasmid (100 ng). Whole cell extracts were prepared after 24 h and assayed for luciferase activity. Average values obtained from four replicates are reported as the mean ± S.E. of luciferase activity by Tax relative to basal expression of the reporter gene in Tax-deficient cells. In the absence of Tax, YopJ-induced changes in the basal activity of either reporter plasmid was essentially negligible (<2-fold). B, 293T cells (1 × 10⁶) were transfected with expression plasmids for Tax (2 μg) and FLAG-tagged YopJ (0.5 μg) as indicated. Endogenous IKK complexes were immunoprecipitated with monoclonal anti-IKKβ antibodies (PharMingen) and assayed for IκB kinase activity as described in the Fig. 2 legend (top panel). IKKβ protein levels were monitored by immunoblotting with polyclonal anti-IKKβ antibodies (second panel). Levels of Tax and YopJ protein expression were determined by immunoblotting cytoplasmic extracts with anti-Tax or anti-FLAG antibodies (lower two panels). C, 293T cells (1 × 10⁶) were transfected with vectors for HA-tagged IKK (25 ng), Tax (0.5 μg), human IKKγ (T7-tagged, 25 ng), and YopJ (100 ng). Cells were radiolabeled with [32P]orthophosphate for 8 h. Ectopic IKKβ complexes were isolated using anti-HA antibodies, washed at high stringency, and fractionated by SDS-PAGE. Resolved proteins were subjected to sequential autoradiography (top panel) and immunoblotting (middle and bottom panels).
or IKKβ.SA. These complexes were then monitored for IkB kinase activity in vitro using a recombinant IkBa substrate (GST-IkBα). As shown in Fig. 2C, upper panel, Tax potently induced the catalytic activity of wild-type IKKβ via an IKKγ-dependent mechanism (lanes 1–3). In contrast, we were unable to detect IkB kinase activity in IKKβ.SA immunoprecipitates (lanes 4–6). These differences in Tax responsiveness were significant, because wild-type IKKβ and IKKβ.SA were comparably expressed at the protein level (lower panel). Given that IKKβ.SA escapes from phosphorylation in Tax-expressing cells (Fig. 2B), we conclude that Tax-induced phosphorylation of the IKKβ catalytic subunit is required for acquisition of constitutive IkB kinase activity.

YopJ Interferes with TaxIKK Signaling—The bacterial virulence factor YopJ binds to multiple MAP2K proteins and interferes with T loop phosphorylation (7). In keeping with the structural link between MAP2K and IKK proteins, YopJ also forms complexes with IKKβ (7). To determine whether YopJ affects the Tax/NF-κB signaling axis, 293T cells were transfected with an NF-κB reporter plasmid (NF-κB-Luc) along with expression vectors for Tax and YopJ. As shown in Fig. 3A, left panel, Tax potently stimulated NF-κB-directed transcription in the absence of YopJ. However, coexpression with YopJ blocked this Tax response in a dose-dependent fashion. In contrast, YopJ failed to inhibit Tax-induced activation of the HTLV1 LTR (right panel), which involves the transcriptional action of CREB/ATF rather than NF-κB (8).

To examine the mechanism of YopJ action on the Tax/NF-κB axis, 293T cells were transfected with vectors for Tax and YopJ, either alone or in combination. We then purified endogenous IKK complexes from recipient cells and monitored them for IkB kinase activity. As shown in Fig. 3B, top panel, Tax potently induced the catalytic activity of IKK in the absence of YopJ (lanes 1 and 2). In contrast, endogenous IKK complexes isolated from cells coexpressing Tax and YopJ failed to affect GST-IkBα phosphorylation (lane 4). Immunoblotting experiments confirmed that comparable amounts of endogenous IKKβ were co-immunoprecipitated under each condition and that YopJ and Tax were both expressed efficiently (lower two panels).

To determine whether YopJ interferes with Tax-induced phosphorylation of IKK, 293T cells expressing ectopic IKK, Tax, and YopJ were radiolabeled with [32P]orthophosphate. We then isolated IKKβ complexes and analyzed their phosphoprotein content. As shown in Fig. 3C, top panel, phosphorylation of IKKβ and IKKγ was significantly increased in the presence of Tax relative to the level of subunit radiolabeling detected in Tax-deficient cells (lanes 3 and 4). Coexpression with YopJ completely blocked this Tax-dependent increase in IKK phosphorylation (lane 5). Given the capacity of YopJ to prevent T loop phosphorylation (7), these findings provide further evidence indicating that Ser-177 and Ser-181 in the T loop of IKKβ function as Tax-responsive phosphoacceptors.

Phosphorylation of IKKγ by IKKβ—The finding that IKKγ phosphorylation induced by Tax is dependent on the catalytic activity of IKKβ (Figs. 1–3) led us to hypothesize that IKKγ is a substrate of IKKβ. To test this possibility, 293T cells were transfected with an expression vector encoding a constitutively active mutant of IKKβ that contained glutamic acid substitutions at Ser-177 and Ser-181 (IKKβ.SA) (10). IKKβ.SA immunoprecipitates derived from these transfectants were incubated with [γ-32P]ATP and a substrate containing GST fused to full-length IKKγ (GST-IKKγ). As shown in Fig. 4 (top, lane 2), GST-IKKγ phosphorylating activity was readily detected in IKKβ.SA immunocomplexes. Removal of the IKKγ sequences from the GST-IKKγ fusion protein eliminated phosphoryl group transfer, thus confirming specificity (middle, lane 2). IKKγ kinase activity was not detected in immunoprecipitates derived from cells expressing a kinase-deficient mutant of IKKβ (IKKβ.SA) (top, lane 3), indicating that phosphorylation of IKKγ is dependent on the catalytic function of IKKβ. Moreover, the IKKγ kinase activity detected in IKKβ.SA immunocomplexes was retained after high stringency washing with 3 M urea (top, lane 5), excluding the involvement of a kinase that associates loosely with IKKβ.

In summary, our data indicate that the assembly of TaxIKK complexes leads to chronic phosphorylation of IKKβ and IKKγ. Activation of IKKβ by Tax appears to involve a kinase that phosphorylates the T loop of IKKβ at Ser-177 and Ser-181. Given that kinase-dead mutants of IKKβ are defective for Tax-induced phosphorylation, this kinase may be IKKγ. Point mutations in IKKβ that disrupt its catalytic function also prevent Tax-induced phosphorylation of IKKγ, suggesting that IKKγ is phosphorylated by IKKβ within the same complex. In keeping with this proposal, IKKβ has the capacity to phosphorylate a recombinant IKKγ substrate in vitro. As such, further studies are warranted to define whether the phosphorylation status of IKKγ affects the temporal regulation of IkB kinase activity.

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