Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 27, Issue of July 6, pp. 24445–24448, 2001

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

Received for publication, October 31, 2000, and in revised form, March 23, 2001
Published, JBC Papers in Press, April 26, 2001,
DOI 10.1074/jbc.C000777200

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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 IKKβ catalytic subunit and IKKγ 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 IKKβ 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 IKKβ that contain glutamic acid substitutions at Ser-177 and Ser-181 have the capacity to phosphorylate a recombinant IKKγ substrate *in vitro*. We conclude that Tax-induced phosphorylation of IKKβ is required for IKKβ activation, phosphoryl group transfer to IKKγ, 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 IκBα, an inhibitory subunit of NF-κB, and an inducible IκB kinase called IKK[1] (2). In response to immune system cues such as the cytokine tumor necrosis factor-α (TNF), IKK phosphorylates IκBα at Ser-32 and Ser-36 (2). In turn, phosphorylated IκBα is degraded and NF-κB translocates to the nucleus (2).

The most well-characterized form of IKK contains two catalytic subunits, termed IKKα and IKKβ, and a regulatory subunit called IKKγ (NEMO) (2). In response to TNF, IKKβ is rapidly phosphorylated, activated, and down-regulated within 30 min (3). The relevant phosphoacceptors in IKKβ 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 IKKγ, which directs the assembly of Tax·IKK complexes (5). Tax also binds to and activates MEKK1, a MAP3K that phosphorylates IKK *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 IKKβ and IKKγ 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 IKKβ inhibit Tax-directed phosphorylation of both subunits and block the chronic stimulatory effects of Tax on IκB kinase activity. Moreover, Tax-induced phosphorylation of IKKβ and IKKγ is antagonized by the bacterial protein YopJ, a potent *in vivo* inhibitor of T loop phosphorylation and IKKβ catalytic activity (7). The finding that Tax-induced phosphorylation of IKKγ is contingent upon IκB kinase activity may reflect IKKβ-mediated phosphorylation of IKKγ within individual Tax·IKK signaling complexes. Consistent with this finding, constitutively active forms of IKKβ have the capacity to phosphorylate a recombinant IKKγ substrate *in vitro*.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal anti-IKK 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 IKKβ (13) was subcloned into pCMV4 (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 (1×10^6) 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 β-mercaptoethanol and the supplements listed above.

* This study was supported by National Institutes of Health Grant RO1 CA82556 (to D. W. B.). 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: IKK, IκB kinase; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; HTLV1, human T-cell leukemia virus type 1; LTR, long terminal repeat; MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TNF, tumor necrosis factor-α.

Reporter gene activity was determined using a Promega Luciferase Assay Kit and a Turner Designs luminometer. All data were normalized to the activity of a cotransfected β-galactosidase expression vector.

Subcellular Fractionation and Biochemical Analyzes—Cytoplasmic extracts were prepared as described (18). Microcystin (1 μM; Alexis Biochemical) was included in the lysis buffer for isolation of endogenous IKKs. Immunoprecipitations were performed in the presence of ELB buffer (18). Kinase activity was measured (18) in reaction mixtures containing ATP (10 μM), [γ-^{32}P]ATP (5 μCi), and recombinant glutathione *S*-transferase protein fused to either a fragment of IκBα (amino acids 1–54; GST·IκBα) or full-length IKKγ (GST·IKKγ) (19). To measure IKK phosphorylation *in vitro*, reaction mixtures contained 2.5 μM ATP and lacked recombinant substrate. Radiolabeled products were washed with RIPA buffer (150 mM NaCl, 10 mM sodium phosphate pH 7.2, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40), fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Proteins were analyzed by immunoblotting using an enhanced chemiluminescence system (Pierce) (18).

Metabolic Radiolabeling—Following transfection (18 h), HeLa and 293T cells were labeled for 8 h with [^{32}P]orthophosphate (1 mCi/ml; ICN) in phosphate-free DMEM (Mediatech). Immunocomplexes were washed with ELB buffer containing 0.5 M NaCl and 1 M urea, followed by RIPA buffer. Jurkat and MT-2 T cells were labeled in phosphate-free DMEM for 8 h with [^{32}P]orthophosphate (2 mCi/ml). Cytoplasmic extracts were precleared with anti-FLAG M2 antibodies prior to immunoprecipitation with anti-IKKγ antibodies. Resultant complexes were washed with ELB buffer containing 2 M urea followed by RIPA buffer. Phosphoproteins were resolved by SDS-PAGE, transferred to PVDF membranes, and analyzed by autoradiography and immunoblotting.

RESULTS AND DISCUSSION

Tax-dependent Phosphorylation of IKK in Vitro—Prior studies have established that IKKγ directs the assembly of Tax·IKK complexes (5). However, the biochemical mechanism by which Tax stimulates IKKβ catalytic activity in these complexes remains unknown. To explore the role of phosphorylation in Tax·IKK signaling, HeLa cells were transfected with combinations of expression vectors for IKKβ, IKKγ, and wild-type Tax. Parallel transfections were performed with mutants of Tax that are selectively defective for either CREB/ATF (Tax-M47) 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-^{32}P]ATP. As shown in Fig. 1*A* (*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 TNF (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. 1*B*) or Tax (Fig. 1*C*) immunoprecipitates for *in vitro* kinase assays. As shown in Fig. 1*B, 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. 1*C*), 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 [^{32}P]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. 2*A*, IKKβ 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

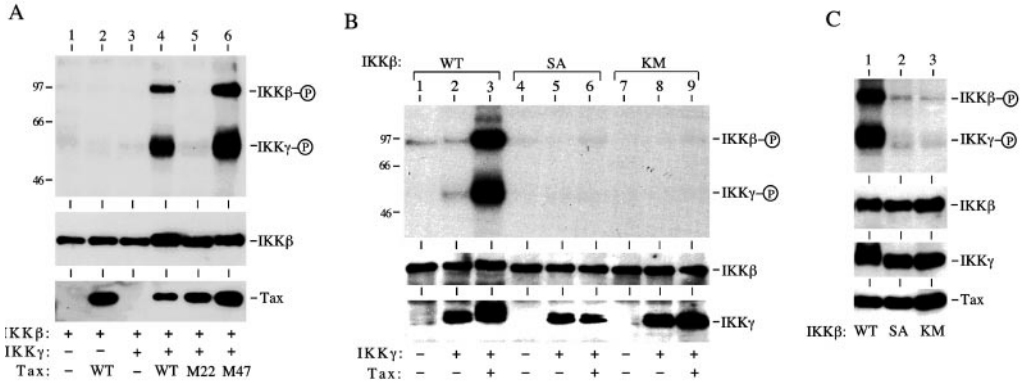

FIG. 1. **Tax-dependent phosphorylation of IKK *in vitro*.** *A*, HeLa cells (2×10^6) were transfected with vectors for FLAG-tagged IKKβ (50 ng), human IKKγ (T7 epitope-tagged, 200 ng), and either wild-type Tax (200 ng), Tax-M22 (600 ng), or Tax-M47 (200 ng). Cytoplasmic extracts were immunoprecipitated with either anti-FLAG M2-agarose beads (*top* and *middle panels*) or monoclonal anti-Tax antibodies (*bottom panel*). IKKβ complexes were subjected to *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. Radiolabeled proteins were resolved by SDS-PAGE and analyzed by autoradiography (*top panel*) and immunoblotting with IKKβ-specific antibodies (*middle panel*). Tax was detected by immunoblotting with polyclonal anti-Tax antibodies (*bottom panel*). *B*, HeLa cells (1×10^6) were transfected with vectors for Tax (100 ng), human IKKγ (T7-tagged, 100 ng), and FLAG-tagged wild-type IKKβ (*WT*), IKKβ.SA, or IKKβ.KM (25 ng each). Ectopic IKKβ was isolated with anti-FLAG M2-agarose beads and subjected to *in vitro* kinase assays as described in *A* (*top panel*). IKK protein levels were determined by immunoblotting with subunit-specific antibodies (*middle* and *bottom panel*). *C*, HeLa cells were transfected as described in *B*. Tax complexes were prepared with monoclonal anti-Tax antibodies and subjected to *in vitro* kinase assays as described in *A* (*top panel*). Relative protein levels were determined by immunoblotting (*lower panels*).

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 [^{32}P]orthophosphate. Ectopic IKKβ was isolated by immunoprecipitation, fractionated by SDS-PAGE, and analyzed by autoradiography. As shown in Fig. 2*B, top panel*, Tax induced significant phosphorylation of IKKβ and IKKγ in cells programmed with the wild-type catalytic subunit (*lanes 1* and *2*). In contrast, Tax-directed phosphorylation of both subunits was blocked in cells expressing either IKKβ.SA (*lanes 3* and *4*), which has alanine replacements at Ser-177/Ser-181, or the kinase-dead mutant IKKβ.KM (*lanes 5* and *6*). Comparable amounts of IKKβ and IKKγ protein were detected in all of the samples analyzed (Fig. 2*B, middle* and *lower panels*). These *in vivo* results correlated strongly with the *in vitro* phosphorylation data shown in Fig. 1*B*.

To explore the functional consequences of IKKβ phosphorylation, IKKβ complexes were immunopurified from HeLa cell transfectants expressing Tax, IKKγ, and either wild-type IKKβ

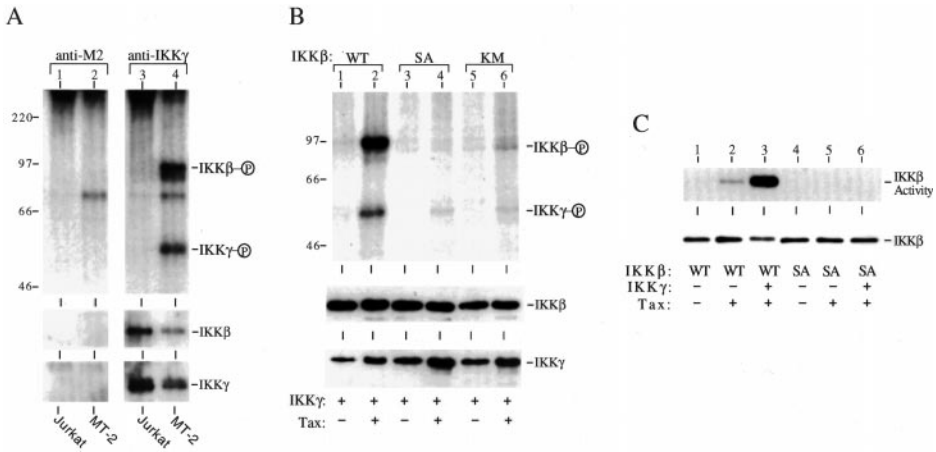

FIG. 2. **Tax induces phosphorylation of IKKβ and IKKγ *in vivo*.** *A*, Jurkat and MT-2 T cells were radiolabeled with [^{32}P]orthophosphate for 8 h. Cytoplasmic extracts (400 μg) were subjected to immunoprecipitation with the indicated monoclonal antibodies. Resultant complexes were fractionated by SDS-PAGE and analyzed for ^{32}P incorporation (*top panel*). IKK protein content was determined by immunoblotting with subunit-specific antibodies (*middle* and *lower panels*). *B*, HeLa cells (1×10^6) 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 IKKβ.KM (50 ng each). Cells were radiolabeled with [^{32}P]orthophosphate for 8 h. Ectopic IKKβ 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-^{32}P]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*).

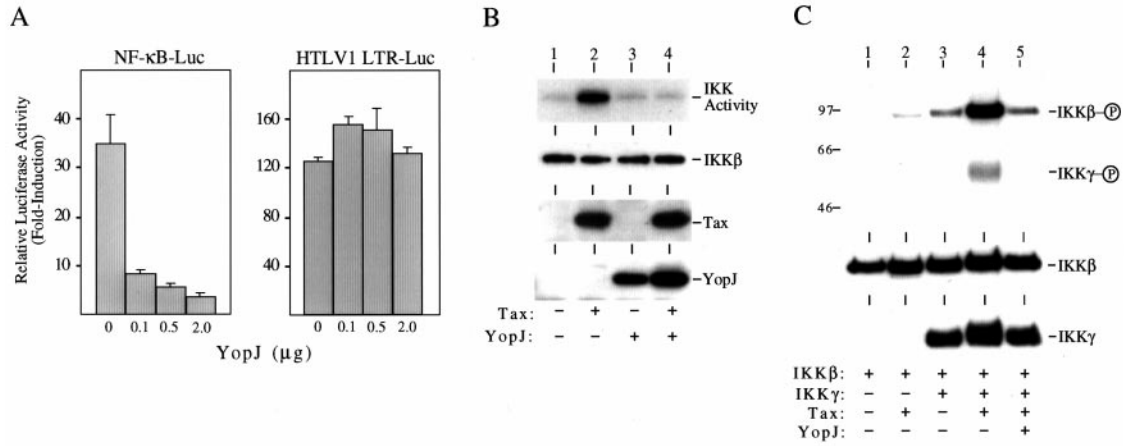

FIG. 3. **YopJ prevents Tax-induced activation of IKK and NF-κB.** *A*, 293T cells (5×10^5) were transfected with a Tax expression vector (1 μg), the indicated amounts of an effector plasmid encoding FLAG-tagged YopJ, and either the NF-κB-Luc or HTLV1 LTR-Luc reporter plasmid (100 ng). Whole cell extracts were prepared after 24 h and assayed for luciferase activity. Average values obtained from four replicates are reported as the mean -fold induction ($\pm$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 (1×10^6) 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-IKKγ 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 (1×10^6) 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 [^{32}P]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*).

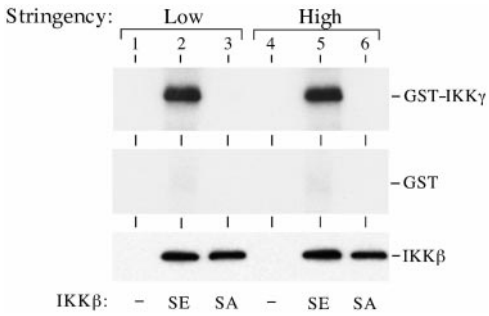

FIG. 4. **Phosphorylation of IKKγ by IKKβ.** 293T cells (0.5×10^6) were transfected with expression plasmids for FLAG-tagged IKKβ.SE (25 ng) or IKKβ.SA (50 ng). Ectopic IKKβ was isolated with anti-FLAG M2-agarose beads and washed under low (250 mM NaCl) or high (250 mM NaCl, 3 M urea) stringency conditions. Resultant immunocomplexes were incubated with either GST·IKKγ (*top panel*) or GST (*middle panel*) for 30 min and then assayed for kinase activity in the presence of [γ-^{32}P]ATP. Relative levels of IKKβ protein were determined by immunoblotting (*bottom panel*).

or IKKβ.SA. These complexes were then monitored for IκB kinase activity *in vitro* using a recombinant IκBα substrate (GST-IκBα). As shown in Fig. 2*C, 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 IκB 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. 2*B*), we conclude that Tax-induced phosphorylation of the IKKβ catalytic subunit is required for acquisition of constitutive IκB kinase activity.

YopJ Interferes with Tax·IKK 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. 3*A, 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 IκB kinase activity. As shown in Fig. 3*B, 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·IκBα 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 [^{32}P]orthophosphate. We then isolated IKKβ complexes and analyzed their phosphoprotein content. As shown in Fig. 3*C, 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.SE) (10). IKKβ.SE immunoprecipitates derived from these transfectants were incubated with [γ-^{32}P]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.SE 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.SE 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 Tax·IKK 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 IKKβ. 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 IκB kinase activity.

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