In Vitro Activation of the IkB Kinase Complex by Human T-cell Leukemia Virus Type-1 Tax

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Human T-cell leukemia virus type-I expresses Tax, a 40-kDa oncoprotein that activates IkB kinase (IKK), resulting in constitutive activation of NFkB. Herein, we have developed an in vitro signaling assay to analyze IKK complex activation by recombinant Tax. Using this assay in combination with reporter assays, we demonstrate that Tax-mediated activation of IKK is independent of phosphatases. We show that sustained activation of the Tax-mediated activation of the NFkB pathway is dependent on an intact Hsp90-IKK complex. By acetylating and thereby preventing activation of the IKK complex by the Yersinia effector YopJ, we demonstrate that Tax-mediated activation of the IKK complex requires a phosphorylation step. Our characterization of an in vitro signaling assay system for the mechanism of Tax-mediated activation of the IKK complex with a variety of mutants and inhibitors results in a working model for the biochemical mechanism of Tax-induced activation.

The first identified pathogenic retrovirus, human T-cell leukemia virus type-I, the causal agent for adult T-cell leukemia, expresses Tax, a 40-kDa phospho-oncoprotein, that plays a pivotal role in the growth and transformation of T-cells (1). Tax chronically stimulates the IkB kinase (IKK) complex via NEMO/IKKγ, resulting in sustained phosphorylation and degradation of IkB and activation of NFkB (2–4). Tax, unlike all the other upstream stimuli such as NFkB-inducing kinase, MEK kinase 1, and transforming growth factor β-activated kinase 1, is not a kinase that phosphorylates and activates the IKK complex. It activates the complex by direct interaction via a mechanism not clearly understood. It has been proposed that Tax activates the NFkB pathway by inducing a conformational change onto IKK (5).

Previous studies have identified several binding partners for Tax, such as MEK kinase 1 (6) and IKKγ in the IKK complex (3). The catalytic subunit of the serine/threonine protein phosphatase 2A (PP2A) was recently identified to bind Tax directly, and it was demonstrated that Tax forms a ternary complex together with IKKγ and PP2A (7). The role of PP2A in inhibiting IKK activation has been established in the past (8). Based on these studies, two models have been proposed to explain the regulation of the IKK complex by Tax in conjunction with PP2A (7, 9). According to one model, Tax binding to PP2A relieves negative inhibition on the IKK complex, resulting in an active IKK complex (7). In contrast, another group has demonstrated that PP2A positively regulates IkB kinase signaling, i.e. IKK–PP2A complexes are essential for IKK activation (9). This gives rise to a second model wherein Tax-mediated activation of IKK requires the association of active PP2A with the IKK complex (9).

In contrast to the viral pathogen human T-cell leukemia virus type-I, the bacterial pathogen Yersinia pestis, the causal agent for bubonic plague, inhibits the NFkB pathway (10, 11). Yersinia species use the bacterial virulence factor YopJ to block all MAPK and NFkB pathways at a common point (11, 12). Recent studies revealed that YopJ functions as an acetyltransferase (13, 14). It blocks the activation of all MAPK kinases, including IKKβ, by the addition of an acetyl group to the highly conserved serine and threonine residues in the activation loop of the kinase. Acetylation of these residues by YopJ prevents the activation of these kinases by inhibiting phosphorylation. Previously, overexpression studies have shown that YopJ inhibits Tax-mediated activation of the IKK complex (15).

To decipher the requirements of Tax-induced NFkB signaling, we have developed an in vitro signaling assay to analyze the activation of the IKK complex by Tax. The assay utilizes wild type and mutant recombinant Tax proteins, an S100 lysate containing unstimulated IKK complex, and a readout for the activation of the NFkB pathway, phospho-IkB. The mutant Tax proteins that we have used in our assays include M22, H41Q, H43Q, and K85N. Fu et al. (7) demonstrated that the ability to induce NFkB activation is abrogated in the M22 mutant whereas the other three mutants are defective in binding PP2A and also fail to activate the NFkB pathway. Based on their study, they proposed that binding of Tax to PP2A is essential for NFkB activation. Herein, we find that activation of the NFkB pathway by recombinant Tax does not require binding to or the activity of PP2A but is dependent on an intact Hsp90-IKK complex. Recombinant Tax is unable to activate the IKK complex in an
Tax Activation of IKK Complex

S100 lysate that contains the acetyltransferase YopJ. Based on results from transcription reporter assays and our in vitro signaling system, we propose that the activation of the IKK complex by human T-cell leukemia virus type-1 Tax requires both a signaling complex containing functional chaperone Hsp90 and an activation loop on IKKB that is required for phosphorylation. This in vitro signaling assay will thus allow us to dissect the mechanism of Tax-dependent activation of IKK.

MATERIALS AND METHODS

Plasmids and Reagents—Tax was cloned into pET28a and pcDNA3 vectors using 5′-BamH1 and 3′-Xho1. The four Tax mutant constructs, M22, H41Q, H43Q, and K85N, in pET28a and pcDNA3 vectors were created using the Stratagene mutagenesis kit with the following pair of oligonucleotides: M22, 5′-ACC CTG GAG CAC CTC CCA AGC GCG TCT TTG CCA GAC CCC GGA CTC, M22, 3′-GAG TCC GGG TCG TGG AAA AGA CGC GCT TGG GAG GTG CTC CCC AAG GGT; H41Q, 5′-GGA CTA TGT TCG GCC CGC CTA CAG GTG CAC GCC CTA CTG GCC ACC, H41Q, 3′-GGT GGC CAG TAG GTG ACG CTG TAG GCC GGC CGA ACA TAG TCC; H43Q, 5′-GGA CTA TGT TCG GCC CGC CTA CAT GTG CAG GCC CTA CTG GCC ACC, H43Q, 3′-GGT GGC CAG TAG GTG ACG CTG TAG GCC GGC CGA ACA TAG TCC; H43Q, 5′-TTC CCC ACC CAG AGA ACC TCT AAT ACC AAT GTC CTG ACC CCG, K85N, 3′-GGG GTC TGG AAA AGA CGC GCT TGG GAG GTG CTG AGC CCC AAG GGT; H11032/H9252-H11032/H10132-BamH1 and 3′-H11032/H10132/Xho1. The four Tax mutants were expressed as His-tagged proteins in Rosetta (Invitrogen) cells, grown to an O.D. of 0.6–0.8, and induced at a concentration of 10 mg/ml.

Human T-cell Leukemia Virus Type-1 Tax and/or all the mutants or 1 μM TRAF6 in the presence of an ATP-regenerating system (10 μM ATP, 350 μM creatine kinase) for 10 min at 37 °C. 0.5 μl of Protein A-agarose beads was added to cleared lysates and was incubated with Tax or TRAF6 recombinant proteins. For in vitro activation of the NFκB pathway, HEK293 cells were transfected with pcDNA3-WT-Tax or mutant Tax and pcDNA3-FLAG-1κB in the absence or presence of 100 ng of pSFFV-YopJ-FLAG or pSFFV-YopJC172A-FLAG. 36 h post-transfection, cells were lysed with HNT lysis buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, and 1% Triton X-100) containing protease inhibitor mixture tablet (Roche Applied Science), 1 mM dithiothreitol, 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM sodium vanadate, and 0.5 mM EGTA. Lysates were immunoblotted with anti-phospho-1κB, anti-FLAG, and anti-Tax antibodies.

pNPP Assay—A 1:50 dilution of Hela-S3 cell-free lysate was incubated in the absence or presence of 10 nm okadaic acid. The 96-well microtiter plate assay (Upstate Biotechnology) was carried out in triplicate according to the manufacturer’s description. The absorbance at wavelength 410 nm was read using a FluoStar Optima (BMG Labtech).

In Vitro Assay Using Recombinant Tax—Cleared lysates (10 mg/ml) were incubated with or without 2 μM recombinant WT Tax and/or all the mutants or 1 μM TRAF6 in the presence of an ATP-regenerating system (10× stock: 10 mM ATP, 350 mM creatine phosphate, 20 mM Hepes, pH 7.2, 10 mM MgCl2, and 500 μg/ml creatine kinase) for 10 min at 37 °C. 0.5 μM okadaic acid and 2 μM geldanamycin were added to cleared lysates before the addition of the recombinant protein. Reactions were terminated by addition of 5× SDS sample buffer. Proteins from the reaction samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. IKK activation was detected by immunoblotting with anti-phospho-1κB antibody. For in vitro assays using Hsp90-immunodepleted lysate, lysates were incubated with anti-Hsp90 antibody for 1 h at 4 °C, followed by incubation with 30 μl of Protein A-agarose beads for 30 min at 4 °C. This step was performed twice to ensure complete immunodepletion of Hsp90. The lysates were finally incubated with Tax or TRAF6 recombinant proteins. In vitro kinase assays were performed on immunoprecipitated complexes as previously described using [γ-32P]ATP and GST-IκBα-1(52) as the recombinant substrate (11).

RESULTS

In Vitro Activation of the NFκB Pathway by Recombinant Human T-cell Leukemia Virus Type-1 Tax—We previously established an assay to study the activation of MAPK and NFκB pathways.
signaling pathways in vitro (13). The assay is initiated by the addition of a partially purified activator of a signaling pathway to an S100 lysate in the presence of an ATP-regenerating system, followed by termination of the reaction by addition of SDS sample buffer. The activation of the signaling pathway is assessed by analysis of a downstream indicator by immunoblotting for a phosphorylated protein. Herein, we analyzed the activation of the IKK complex (Fig. 2C). As expected, only complexes isolated from the Tax-activated lysates were able to phosphorylate GST-1xBo (Fig. 2B and C).

**Tax-mediated Activation of NFxB Pathway Is Independent of Binding to Phosphatases**—To determine the role of phosphatases in the activation of the NFxB pathway by Tax, several previously described mutants of Tax (7) were used in our *in vivo* and *in vitro* signaling assays. The effect on the activation of the NFxB pathway by wild type and mutant Tax proteins was first analyzed using transfection-based luciferase assays with an NFxB luciferase reporter (Fig. 3A). The activation of the NFxB pathway by the various Tax mutants was also analyzed by immunoblotting cell lysates with anti-phospho-1xB antibody (Fig. 3B). We observed that the M22 mutant, which has previously been described as the inactive form of Tax, fails to induce activation of the NFxB pathway, and these findings are consistent with previous studies in which this mutant was shown to be unable to activate the NFxB pathway (Fig. 3, A and B) (7). Tax mutants H41Q, H43Q, and
K85N activated the NFκB pathway to varying levels, based on both the luciferase assays and the immunoblotting for phospho-IκB (Fig. 3, A and B). For the latter experiment, cells were constitutively expressed with FLAG-IκB to detect phosphorylation of exogenous IκB, because endogenous IκB is degraded upon activation of the NFκB pathway (13). The mutants H43Q and K85N were not detected using the anti-Tax antibody because of their decreased expression levels in the mammalian system (Fig. 3B) (17). Because H41Q, H43Q, and K85N, which are defective in binding PP2A, were able to activate the NFκB pathway, the binding of PP2A to Tax does not appear to be essential for Tax-mediated activation of the IKK complex (7).

The Tax mutants were also expressed as recombinant His-tagged proteins in bacteria and purified using nickel affinity chromatography (Fig. 1A). The anti-Tax antibody was able to immunoreact with all the recombinant proteins (Fig. 1B). Consistent with previous in vivo observations, addition of the recombinant inactive mutant form of Tax (M22) to the in vitro signaling system was unable to induce phosphorylation of IκB (Fig. 4A, lane 5). However, addition of the three recombinant mutant forms of Tax including H41Q, H43Q, and K85N, which are unable to bind PP2A, resulted in activation of the NFκB pathway as indicated by phosphorylation of IκB in vitro, albeit with varying potency (Fig. 4A, lanes 3, 4, and 6). The profiles of partially purified wild type Tax and the inactive mutant M22 proteins (Fig. 1, lanes 1 and 2) appear the same, supporting the proposal that differences in the activities can be attributed to
the mutated amino acids in the various Tax proteins. Overall, these observations do not support the first model, in that binding of PP2A to Tax is not essential for Tax activation of the IKK complex (7).

**Tax-mediated Activation of the NFκB Pathway Is Independent of Active Phosphatases**—To further assess whether PP2A plays a positive role in the in vitro activation of the IKK complex by Tax, the in vitro activation of the NFκB pathway was analyzed in the presence of okadaic acid (OA). Addition of 10 nM OA (or 500 nM, data not shown) did not alter the ability of wild type Tax to activate the IKK complex as observed by phosphorylation of IκB (Fig. 4B, lane 4). At this very low concentration of OA (10 nM), PP2A activity is effectively repressed in the lysate (Fig. 4C). Therefore, when PP2A activity is inhibited, Tax is still able to activate the IKK complex. As expected, there was no change in IκB phosphorylation levels upon addition of TRAF6 to the lysate in the presence of OA (Fig. 4B, lane 6).

Based on these observations, Tax can activate the IKK complex independent of PP2A activity, thereby discounting the second model that predicted that the interaction between IKK and PP2A is essential for Tax to be able to activate the IKK complex (9).

**Hsp90 Is Essential for Tax-mediated Activation of the IKK Complex**—Studies were initiated to further understand the requirements for activation of the IKK complex by Tax. The in vitro signaling system recapitulated the activation of the NFκB pathway by Tax and also demonstrated that Tax-mediated IKK activation is independent of phosphatases. It is possible that by binding to IKKγ, Tax is inducing a conformational change that results in the activation of the IKK complex (5). Hsp90, which is known to maintain the structural integrity of protein complexes (18), is an integral component of the IKK complex (19).

To test whether Hsp90 plays a role in Tax-mediated activation of the NFκB pathway in vitro, geldanamycin (GA), an Hsp90-specific inhibitor, was used in the in vitro signaling system. Upon addition of 2 μM GA to the in vitro signaling assay, it was observed that Tax could no longer activate IKK as indicated by the lack of IκB phosphorylation (Fig. 5A, lane 4). GA also inhibited the TRAF6-dependent activation of the IKK complex (Fig. 5A, lane 7). This is consistent with previous observations where GA-dependent Hsp90 inhibition has been shown to interfere with IKK activation (19). To further assess the effect of Hsp90 on Tax-mediated NFκB activation, we used lysate, immunodepleted for Hsp90 (Fig. 5B), in our in vitro cell-free signaling assay. Addition of recombinant Tax and TRAF6 proteins to Hsp90-immunodepleted lysate failed to activate the NFκB pathway as shown by immunoblotting against phospho-IκB (Fig. 5C, lanes 5 and 6). Because Hsp90 is known to maintain the structural integrity of protein complexes, these observations support a new model that a preformed native complex requiring active Hsp90 is essential for the activation of the IKK complex by Tax. This supports the proposed model that binding of Tax to IKKγ in the IKK complex causes a conformational change that induces autoactivation of the kinases in the complex (5).

**Tax Cannot Bypass Inhibition of the IKK Complex by YopJ Acetylation**—Tax is one of the upstream activators of the IKK complex; however, the mechanism by which it activates IKK has not been completely deciphered. The effector protein YopJ from *Yersinia* was recently shown to inhibit MAPK kinase and IKK activation by acetylating the conserved serine and threonine residues in the activation loop of the kinase (13, 14, 20). Consistent with previous findings, YopJ inhibited IKK activation by Tax, as shown by using a NFκB luciferase reporter (Fig. 6A) (15). The catalytically inactive mutant YopJC172A, however, had no effect on Tax-mediated activation of NFκB (Fig. 6A). Transfection experiments with YopJ and Tax followed by immunoblotting with antibody against phospho-IκB further confirmed these results because YopJ, but not YopJC172A, inhibited Tax-mediated IκB phosphorylation (Fig. 6B). As before, cells constitutively expressed FLAG-IκB to detect phosphorylation of exogenous IκB.

**YopJ Inhibits in Vitro Activation of IKK by Tax**—To gain insight into the mechanism used by Tax, in vitro assay of Tax-mediated IKK activation was utilized. 10 mg/ml membrane-cleared lysate was prepared from cells transfected with vector (V), YopJ (J), or YopJC172A (C/A) plasmids (13). Recombinant WT Tax was then used to activate these lysates. Tax was able to phosphorylate endogenous IκB in both V- and C/A-transfected cell lysates (Fig. 6C). By contrast and as expected, Tax had lost its ability to phosphorylate IκB from cells expressing YopJ. The results from this in vitro assay confirm the above in vivo results and are in

![FIGURE 5. Inhibition of Tax-mediated activation of the NFκB pathway by geldanamycin. A, in vitro activation of the IKK complex by buffer (lanes 1 and 2), Tax (lanes 3 and 4), or TRAF6 (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of geldanamycin (GA). Thin lines denote where lanes have been deleted from the scanned image. Activation of the IKK complex was detected by immunoblotting with anti-phospho-IκB antibody. B, cell-free lysates (1 mg/ml) were immunodepleted with Hsp90 by performing two successive rounds of immunoprecipitation with anti-Hsp90 antibody (1:100). The levels of Hsp90 and IKKβ in these lysates were assessed by immunoblotting against anti-Hsp90 (upper panel) and anti-IKKβ antibodies. C, lysates immunodepleted with Hsp90 (lanes 4–6) or not (lanes 1–3) were incubated with 2 μM Tax and 1 μM TRAF6 for 10 min at 37 °C. Samples were subjected to SDS-PAGE followed by immunoblotting with anti-phospho-IκB antibody. The data shown are representative of three independent experiments.](image-url)
**Tax Activation of IKK Complex**

**A**

![Graph showing Tax Activation of IKK Complex](image)

**B**

![Figure 6. Activation of the NFκB pathway by Tax is inhibited by the acetyltransferase YopJ. A, activation of an NFκB luciferase reporter gene by Tax. HEK293 cells were transfected with an empty vector (Vector) or wild type Tax (pCDNA3-Tax) in the presence of an empty vector (V), YopJ (J), or catalytically inactive YopJC172A (C/A), the 5xNFκB luciferase reporter, and pRSV- Renilla (to serve as the internal standard control) for 24 h. The luciferase assay was performed using Fluostar Optima. B, HEK293 cells were cotransfected with Tax and either empty vector (V), YopJ (J), or catalytically inactive YopJC172A (C/A) in the presence or absence of FLAG-IκB followed by immunoblotting cell lysates with anti-phospho-IκB antibody and anti-IκB antibody. The asterisk indicates transfected FLAG-IκB. C, in vitro activation of the IKK complex by Tax (+, 0.8 μM; ++, 1.6 μM) in lysates isolated from cells containing empty vector (V), YopJ (J), or catalytically inactive YopJC172A (C/A). Activation of IKK complex was detected by immunoblotting with anti-phospho-IκB antibody. The data shown are representative of three independent experiments.**

**C**

![Graph showing Tax Activation of IKK Complex](image)

In accordance with the work by Carter et al. (15). Just like all other upstream stimuli, YopJ is able to block the Tax-mediated activation of the IKK complex.

**DISCUSSION**

In this study, an *in vitro* signaling assay was used to analyze the activation of IKK by Tax. Recombinant Tax, purified from bacteria, was able to efficiently induce IκB phosphorylation in cleared lysates. The phosphorylation profiles of the Tax-activated complexes isolated by immunoprecipitation with IKKα or Tax appear the same, supporting the accepted hypothesis that Tax, IKKα, and the IKKs are parts of the same signaling complex. The PP2A binding-deficient mutants of Tax were also able to activate NFκB signaling, although not as strongly as WT Tax. These observations do not support the model that the binding of PP2A to Tax is essential for Tax-mediated activation of the IKK complex (7). In addition, experiments with okadaic acid added to the *in vitro* assay did not alter the ability of WT Tax to activate the IKK complex and therefore do not support the alternative model that Tax-mediated IKK activation is positively regulated by PP2A (9). By contrast, Tax was unable to activate the NFκB pathway when Hsp90, an integral component of the IKK complex, was inhibited by the addition of geldanamycin to the assay. Binding of Tax to IKKγ in the IKK complex may cause a conformational change that induces autoactivation of the kinases in the complex. As more mutants associated with the other activities of viral Tax are discovered (21), this system can be used to diagnose their role in the activation of IKK.

Both *in vivo* and *in vitro* studies revealed that YopJ blocks the activation of IKK by Tax. YopJ leads to the acetylation of the conserved serine and threonine in the activation loop of kinases, including IKK. When the activation loop residues are acetylated, they can no longer be phosphorylated and the kinase cannot be activated (13, 14, 20). The acetyltransferase activity of YopJ on IKK thus competes against phosphorylation of IKK by upstream kinases. As YopJ inhibits Tax-mediated IKK activation, these studies strongly indicate that phosphorylation of IKK is a key intermediate step in the activation of IKK by Tax.

Our results support previously postulated mechanisms for Tax-mediated activation of the IKK complex, including induction of a conformational change or recruitment of an upstream kinase (5). The *in vitro* signaling system used in this study in combination with a number of inhibitors and activators has been useful for the elucidation of the biochemical mechanism of Tax-induced activation of the IKK complex. Based on these studies, a model is proposed whereby Tax-dependent activation of the IKK complex requires active Hsp90 and phosphorylation of IKK. Future biochemical studies that further dissect this mechanism may reveal other factors that are essential for the activation of the IKK complex by Tax.

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