A Novel NF-κB Pathway Involving IKKβ and p65/RelA Ser-536 Phosphorylation Results in p53 Inhibition in the Absence of NF-κB Transcriptional Activity*

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Nuclear factor κB (NF-κB) plays an important role in regulating cellular transformation and apoptosis. The human T-cell lymphotropic virus type I protein, Tax, which is critical for viral transformation, modulates the transcription of several cellular genes through activation of NF-κB. We have demonstrated previously that Tax inhibits p53 activity through the p65/RelA subunit of NF-κB. We now present evidence that suggests that the upstream kinase IKKβ plays an important role in Tax-induced p53 inhibition through phosphorylation of p65/RelA at Ser-536. First, mouse embryo fibroblast (MEF) IKKβ−/− cells did not support Tax-mediated p53 inhibition, whereas MEFs lacking IKKα allowed Tax inhibition of p53. Second, transfection of IKKβ wild type (WT), but not a kinase-dead mutant, into IKKβ−/− cells rescued p53 inhibition by Tax. Third, the IKKβ-specific inhibitor SC-514 decreased the ability of Tax to inhibit p53. Fourth, we show that phosphorylation of p65/RelA at Ser-536 is important for Tax inhibition of p53 using MEF p65/RelA−/− cells transfected with p65/RelA WT or mutant plasmids. Moreover, Tax induced p65/RelA Ser-536 phosphorylation in WT or IKKα−/− cells but failed to induce the phosphorylation of p65/RelA Ser-536 in IKKβ−/− cells, suggesting a link between IKKβ and p65/RelA phosphorylation. Consistent with this observation, blocking IKKβ kinase activity by SC-514 decreases the phosphorylation of p65/RelA at Ser-536 in the presence of Tax in human T-cell lymphotropic virus type I-transformed cells. Finally, the ability of Tax to inhibit p53 is distinguished from the NF-κB transcription activation pathway. Our work, therefore, describes a novel Tax-NF-κB p65/RelA pathway that functions to inhibit p53 but does not require NF-κB transcription activity.

Human T-cell lymphotropic virus type I (HTLV-I) is the etiologic agent of the aggressive and fatal disease adult T-cell leukemia and the neurodegenerative disease tropical spastic paraparesis/HTLV-I-associated myelopathy (1–4). HTLV-I is also associated with arthritis, uveitis, infective dermatitis, and mild immunosuppression (5–7). HTLV-I encodes a 40-kDa protein, Tax, which plays a key role in viral replication, transformation, and gene regulation. Tax not only activates expression of viral genes via the viral long terminal repeat (LTR) but has also been reported to regulate the expression of a number of cellular genes that encode proteins involved in cell growth and apoptosis (5, 8–10). Several of these genes are regulated by the transcription factor NF-κB (nuclear factor κB). Indeed, the ability of Tax to activate the NF-κB pathway is critical for T-cell immortalization and factor-independent growth (11, 12).

NF-κB is a member of the Rel family of proteins, which includes p50, p65/RelA, c-Rel, p52, and RelB. In most cells, NF-κB is present in the cytoplasm in an inactive form through association with IκB family members (13, 14). Activators mediate a rapid phosphorylation of IκB by IκB kinase (IKK), which results in subsequent ubiquitination and proteolytic degradation. NF-κB is then transported to the nucleus, where it activates transcription of target genes through binding to NF-κB target sequences within the promoter. HTLV-I Tax can induce constitutive NF-κB activation through phosphorylation of both IκBα and IκBβ (15). It has been further demonstrated that IKKγ plays an essential role for Tax mediation of NF-κB activation (16–18). Interaction of Tax with IKKγ facilitates its recruitment to the IKKα/IKKβ complex, inducing kinase activation (19).

Several reports have suggested that phosphorylation of p65/RelA is required for efficient transcriptional activation (20). The sites of phosphorylation and its effect on transcription are, however, complex. For example, Ser-276, situated in the Rel homology domain, can be phosphorylated by the catalytic subunit of protein kinase A as well as by mitogen- and stress-activated protein kinase-1 (23–25) or by the IKK complex in response to TNF (25, 26). In contrast, Ser-529 or Ser-536, located in the transactivation domain, can be phosphorylated by casein kinase II in response to TNF or interleukin-1 (23–25) or by the IKK complex in response to TNF (25, 26), respectively.

Our previous studies have demonstrated that Tax inhibits p53 transcriptional activity through the NF-κB signaling pathway, specifically the p65/RelA subunit (27, 28). Tax induces the p65/RelA subunit of NF-κB to interact with p53, which is stably associated with the transcriptional complex in vivo (28). In this study, we demonstrate that the inhibition of p53 activity is dependent upon phosphorylation of p65/RelA at Ser-536 by the upstream kinase IKKβ. Of interest, inhibition of p53 activity does not require NF-κB transcriptional activity. Our work, therefore, describes a novel Tax-NF-κB p65/RelA pathway that inhibits p53 but does not require NF-κB transcriptional activity.
**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfections**—HTLV-I-transformed C81 cells were grown in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. MEF WT and p65/RelA−/− cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum, 2 mM L-glutamine, and penicillin/streptomycin. MEF WT and p65/RelA−/− cells were described previously. MEF IKKa−/− and IKKβ−/− cells were kindly provided by Dr. Warner Greene (University of California, San Francisco). 293T cells and MEFs were transfected using Effectene transfection reagent (Qiagen) and Lipofectamine Plus transfection reagent (Invitrogen), respectively, as described by the manufacturer.

**Plasmids and Luciferase Assays**—The reporter constructs MDM2-Luc, 4× NF-κB-Luc, HTLV-I LTR-Luc, and the Tax expression plasmid (pCTax) have been described previously (see Ref. 27). Flag-p65/RelA WT and mutants S529A and S536A were provided by Dr. Albert Baldwin, Jr. (University of North Carolina, Chapel Hill). IKKα WT and the kinase-dead (KD) mutant were provided by Dr. Michael Karin (University of California, San Diego). For luciferase assays, cell lysates were prepared 24 h after transfection following the Promega dual luciferase and Tropix GalactoLight assay kit instructions. Cells were treated with the IKKβ-specific inhibitor SC-514 (Calbiochem) for 1 h before transfection. All transfactions included the control plasmid RSV β-galactosidase to control for transfection efficiency.

**Western Blot Analysis**—Cell extracts were prepared by using lysis buffer (50 mM Tris, 120 mM sodium chloride, 5 mM EDTA, 0.5% Nonidet-P40, 50 mM sodium fluoride, and 0.2 mM sodium vanadate). The extracts were incubated on ice for 15 min and centrifuged (10,000 × g) at 4 °C for 10 min, and supernatants were collected. Protein concentrations were determined by Bradford assay (Bio-Rad), and 50–100 μg was separated by electrophoresis on 4–20% Tris-glycine gels (Novex). The proteins were then transferred to polyvinylidene difluoride membranes (Immobilon) and analyzed with the desired antibodies. Anti-p65/RelA and p53 were purchased from Upstate Biotechnology and Oncogene Research Products, respectively. Anti-phospho-p65/RelA (Ser-536) and phospho-IKKα/β (Ser-180/Ser-181) were purchased from Cell Signaling. Anti-IKKα (M-280), IKKα/β (H-470) and actin were purchased from Santa Cruz Biotechnology. Anti-IκBα was purchased from Active Motif. To detect the expression of Tax protein, anti-Tab172 monoclonal antibody was used.

**In Vitro IKK Kinase Assay**—For immunoprecipitation kinase assay, IKK complexes from the cytoplasm were precipitated with anti-IKKβ (Santa Cruz Biotechnology) followed by treatment with 10 ml of Dynal beads-protein G (Dynal Biotech). Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2, 2 mM dithiothreitol, 20 mM ATP, 20 mM β-glycerol phosphate, 20 mM diosidum p-nitrophenyl phosphate, and 0.1 mM sodium orthovanadate).

**RESULTS**

IKKβ, but Not IKKa, Is Important for Tax-mediated p53 Inhibition—We demonstrated previously that Tax inhibition of p53 activity is dependent upon the phosphorylation of IκBα by overexpression of a phosphorylation mutant of IκBα, which inhibits NF-κB activation in a dominant-negative manner (27, 28). Because Tax activates NF-κB through the IKK complex and IKKα and IKKβ phosphorylate IκBα and p65/RelA to activate NF-κB (19, 29, 30), we investigated the role of IKKα and IKKβ in Tax-mediated NF-κB p53 inhibition. WT, IKKa−/−, or IKKβ−/− MEFs were transiently transfected with the p53-responsive MDM2-Luc or the NF-κB-responsive 4× NF-κB-Luc reporter constructs in the absence or presence of Tax (Fig. 1A). The results of these studies demonstrated that Tax inhibited p53 activity in WT and IKKα−/− cells but failed to inhibit p53 transcriptional activity in the IKKβ−/− cells (Fig. 1A). In both WT and IKKα−/− cells, Tax inhibited p53 activity by 75–80%.

In parallel assays, we measured the importance of IKKα and IKKβ in Tax-mediated NF-κB activation. NF-κB transcriptional activity was significantly increased by Tax transfection in WT cells (Fig. 1B, compare lane 1 and 2). In WT MEFs, Tax increased NF-κB promoter activity 4–5-fold. In contrast, Tax failed to activate NF-κB activity in either IKKa−/− or IKKβ−/− cells. The fact that p53 inhibition but not NF-κB activity is observed in the IKKα−/− cells suggests that NF-κB transcriptional activity is not required for p53 inhibition. To further test this hypothesis, the NF-κB-responsive human immunodeficiency virus LTR-β-galactosidase reporter was transfected into IKKa−/− cells in the absence (white) or presence (black) of Tax (0.2 μg). 24 h post-transfection, cells were collected, and luciferase activities were measured. Luciferase values were adjusted for transfection efficiency using RSV β-galactosidase. The graph represents the luciferase activity from three independent experiments. The standard deviation for the three experiments is included. C, Western blot analysis of transfected cells was performed for anti-IKKα, IKKβ (Santa Cruz Biotechnology), and Tax (Tab172).

**FIG. 1.** Tax fails to inhibit p53 activity in mouse embryo fibroblast IKKβ−/− but not in WT or IKKα−/− cells. MEFs were transiently transfected using Lipofectamine Plus reagent (Invitrogen) with 0.2 μg of reporter constructs MDM2-Luc (A) or 4× NF-κB-Luc (B) in the absence (white) or presence (black) of Tax (0.2 μg). 24 h post-transfection, cells were collected, and luciferase activities were measured. Luciferase values were adjusted for transfection efficiency using RSV β-galactosidase. The graph represents the luciferase activity from three independent experiments. The standard deviation for the three experiments is included.
activity was strongly activated by Tax with IKKβ WT but not with the KD mutant (Fig. 2B, lane 4, compare with lane 6). In contrast, transfection of IKKβ WT or KD had little effect on Tax transactivation of the cAMP-response element-binding protein-responsive HTLV-I LTR-Luc (Fig. 2C). Western blot analysis of Tax, IKKβ, and p53 demonstrated that the proteins were expressed at roughly equivalent levels (Fig. 2D). Together, these data suggest that IKKβ kinase activity is important for p53 inhibition by Tax.

From studies recently published by O'Mahony et al. (30) and Gregory et al. (31), in IKKα−/− cells like WT MEFs, p65 is predominantly cytoplasmic. Upon NF-κB stimulation, IκBα is phosphorylated and degraded with a concomitant increase in nuclear p65. p65 nuclear levels are, however, reduced in IKKα−/− compared with wild type cells. These results suggest that although somewhat impaired, the normal upstream signaling and release of p65 is intact in IKKα−/− cells. The defect in NF-κB-responsive promoter activity appears to be at the level of p65 transcriptional activation, perhaps through p65 modification or modification of histones (32, 33).

IKKβ-Specific Inhibitor SC-514 Blocks p53 Inhibition Pathway—To further test this hypothesis, we utilized the IKKβ-specific inhibitor SC-514, which does not inhibit IKKα or other serine-threonine and tyrosine kinases (34). p65/RelA/−/− cells were treated with SC-514 1 h prior to transfection. p65/RelA and Tax plasmid DNAs were transfected with either MDM2-Luc, 4× NF-κB-Luc, or HTLV-I LTR-Luc; cells were maintained for 24 h, and luciferase activities were determined. In the presence of Tax and p65/RelA, p53 activity was inhibited (Fig. 3A, lanes 1–4). SC-514 treatment increased p53 transcriptional activity in a dose-dependent manner in the presence of Tax (Fig. 3A, lanes 4, 6, 8, and 10). In the absence of p65/RelA, p53 activity was not changed by SC-514 with or without Tax (data not shown). As further controls for this experiment, we checked the effect of SC-514 on NF-κB activity. As expected, 4× NF-κB-Luc activity was decreased by SC-514 (Fig. 3B). Interestingly, SC-514 appeared to inhibit NF-κB activity more efficiently in the presence of Tax (Fig. 3B). Reporter gene activity from the HTLV-I LTR-Luc construct, which is regulated through cAMP-response element-binding protein, was not significantly influenced by SC-514 in the absence or presence of Tax (Fig. 3C). Western blot analysis showed that the levels of IKKα/β, Tax, p65/RelA, and p53 were not changed in response to SC-514 treatment (Fig. 3D). The level of phospho-IKKα/β was decreased by SC-514 in a concentration-dependent manner. This decrease must be due to inhibition of IKKα/β autophosphorylation and/or phosphorylation of IKKα by IKKβ (35).

Taken together, the results indicate that IKKβ kinase activity is a key factor for Tax-mediated p53 inhibition.

IKKβ induces Phosphorylation of p65/RelA in the Presence of Tax In Vitro—Because p65/RelA was found to be critical for p53 inhibition and IKKβ has been reported as an upstream kinase, which phosphorlylates NF-κB p65/RelA (36, 37), we tested whether IKKβ activity is linked to phosphorylation of p65/RelA in HTLV-I-transformed cells. In vitro kinase assays were performed with immunoprecipitates from C81 cells following treatment with or without SC-514. Cell lysates were prepared and immunoprecipitated with the anti-IKKβ antibody. Kinase activity was assayed using His6-p65/RelA or glutathione-S-transferase-IκBα, which served as a control substrate in those assays. SC-514 treatment reduced phosphorylation of both p65/RelA and IκBα (Fig. 4A). GelCode staining of the gels demonstrated that comparable amounts of p65/RelA or IκBα were present in the reactions (Fig. 4B). Western blot analysis showed that the level of IKKβ was not significantly changed by SC-514 treatment (Fig. 4C, top panel). Consistent with the results of the in vitro kinase assay, we observed that the level of phospho-IKKβ was reduced in C81 cells treated with SC-514 (Fig. 4C, bottom panel). It is important to note that in the C81 cells, we refer only to IKKβ. We have done Western blots on HTLV-I-transformed C81 cell extracts with antibody specific for IKKα and IKKβ. Consistent with the results reported from Yamaoka’s laboratory (38), IKKβ but not IKKα was detected in these cells. Thus, although we saw both IKKα and IKKβ in the MEF cells, in the C81 cells primarily IKKβ was detected. Together, these results suggest that IKKβ can phosphorylate p65/RelA as well as IκBα in HTLV-I-transformed cells.

Tax Induces Phosphorylation of p65/RelA at Ser-536 in HTLV-I-Transformed Cells—Next, we tested whether IKKβ phosphorylates p65/RelA at Ser-536 (35, 39). C81 cells were transfected into SC-514 with or without Tax (data not shown). As further controls for this experiment, we checked the effect of SC-514 on NF-κB activity. As expected, 4× NF-κB-Luc activity was decreased by SC-514 (Fig. 3B). Interestingly, SC-514 appeared to inhibit NF-κB activity more efficiently in the presence of Tax (Fig. 3B). Reporter gene activity from the HTLV-I LTR-Luc construct, which is regulated through cAMP-response element-binding protein, was not significantly influenced by SC-514 in the absence or presence of Tax (Fig. 3C). Western blot analysis showed that the levels of IKKα/β, Tax, p65/RelA, and p53 were not changed in response to SC-514 treatment (Fig. 3D). The level of phospho-IKKα/β was decreased by SC-514 in a concentration-dependent manner. This decrease must be due to inhibition of IKKα/β autophosphorylation and/or phosphorylation of IKKα by IKKβ (35).
Mouse embryo fibroblast p65/RelA−/− cells were transiently transfected using Lipofectamine Plus reagent (Invitrogen) with SC-514 (10, 50, or 100 μM, Calbiochem) was added to cells 1 h prior to transfection. Cells were harvested 24 h after transfection, and luciferase activity was measured. Luciferase values were adjusted for transfection efficiency using RSV β-galactosidase. The graph represents the luciferase activity from three independent experiments. The standard deviation for the three experiments is included. D, Western blot analysis of transfected cells was performed for IKKa/β (Santa Cruz Biotechnology), phospho-IKKα/β (Cell Signaling), p65/RelA (C-terminal, Upstate Biotechnology), Tax (Tα72), and p53 (Ab1, Oncogene Research Products).

FIG. 3. IKKa/β-specific inhibitor SC-514 rescues Tax inhibition of p53. Mouse embryo fibroblast p65/RelA−/− cells were transiently transfected using Lipofectamine Plus reagent (Invitrogen) with 0.2 μg of reporter constructs MDM2-Luc (A), 4× NF-κB-Luc (B), or HTLV-I LTR-Luc (C) and p65/RelA WT (0.05 μg) in the absence (white) or presence (black) of Tax (0.2 μg). SC-514 (10, 50, or 100 μM, Calbiochem) was added to cells 1 h prior to transfection. Cells were harvested 24 h after transfection, and luciferase activity was measured. Luciferase values were adjusted for transfection efficiency using RSV β-galactosidase. The graph represents the luciferase activity from three independent experiments. The standard deviation for the three experiments is included. D, Western blot analysis of transfected cells was performed for IKKa/β (Santa Cruz Biotechnology), phospho-IKKα/β (Cell Signaling), p65/RelA (C-terminal, Upstate Biotechnology), Tax (Tα72), and p53 (Ab1, Oncogene Research Products).

their original levels. The difference in the kinetics indicates that the turnover of phosphorylated p65 is slower than that of IκBα. Because IKKβ kinase activity is inhibited at 18 h post-treatment (Fig. 4A), the reappearance of phosphorylated IκBα suggests that other kinases substitute for IKKβ and phosphorylate IκBα.

A more compelling argument for IKKβ phosphorylation of p65/RelA in the presence of Tax came from studies in which WT, IKKa−/−, or IKKβ−/− cells were transfected with increasing amounts of Tax for 48 h and cell lysates were prepared. Western blot analysis was performed with antibodies specific for phospho-p65/RelA Ser-536, p65/RelA, Tax, and IκKα/β (Fig. 5B). In WT or IKKa−/− MEFs, Tax expression increased the level of phospho-p65/RelA. In contrast, Tax failed to induce phosphorylation of p65/RelA at Ser-536 in IKKβ−/− MEF cells. These results indicate that IKKβ is essential for Tax-induced p65/RelA Ser-536 phosphorylation. The anti-IKKα/β antibody used in this experiment predominantly reacts with IKKβ and is partially cross-reactive with IκKα. Thus, we could detect IKK in WT and IKKa−/− but not in the IKKβ−/− cells.

Tax-mediated p53 Inhibition Requires p65/RelA Phosphorylation at Ser-536—Given the importance of IKKβ and its ability to phosphorylate p65/RelA, it was interesting to determine whether phosphorylation of p65/RelA plays a role in Tax-mediated p53 inhibition. MEF p65/RelA−/− cells were transfected with p65/RelA WT or the serine to alanine mutants S529A or S536A in the absence or presence of Tax and MDM2-Luc or 4× NF-κB-Luc reporter constructs (Fig. 6). Cell lysates were prepared 24 h after transfection, and luciferase activities were measured. Tax-induced p53 inhibition in the presence of p65/RelA WT or S529A (Fig. 6A, lanes 4 and 6). In contrast, Tax failed to inhibit p53 in the presence of p65/RelA S536A (Fig. 6A, lane 8), suggesting that p65/RelA phosphorylation at Ser-536 is required for Tax-mediated p53 inhibition. The activity of the p65/RelA mutants was also measured using the NF-κB-responsive reporter. The ability of S536A to activate transcription was impaired in the absence or presence of Tax. In contrast, mutant S529A activity was similar to the WT p65/RelA protein (Fig. 6B). Western blot analysis was performed to detect the expression of Tax and p65/RelA (Fig. 6C). p65/RelA WT, S529A, and S536A were expressed at approximately equal levels. Tax expression was roughly equivalent in each of the transfections.

To explore whether Tax regulates p65/RelA phosphorylation at Ser-536, cells were transfected with increasing amounts of Tax. 48 h post-transfection, cell lysates were prepared, and Western blot membranes were analyzed for anti-phospho p65/RelA Ser-536. The results demonstrated that Tax increases the phosphorylation of p65/RelA at Ser-536 (Fig. 6D). The increase in IκBα phosphorylation (Fig. 6D) is consistent with published studies (40, 41) and demonstrates Tax activation of the NF-κB pathway.

DISCUSSION

NF-κB is a key cellular transcription factor, which is activated by a variety of stimuli including TNF, lipopolysaccharide, interleukin-1, and γ-irradiation. In the classical activation cascade, the IKK complex, which is composed of two catalytic subunits, IKKa and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO), is phosphorylated by upstream kinases NF-κB-inducible kinase (42, 43), MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase) (44, 45), or AKT (46, 47). Subsequently, IKK induces the phosphorylation of IκBα, resulting in its ubiquitination and degradation. This allows NF-κB heterodimers, primarily p50/p65, to translocate to the nucleus where they stimulate expression of target genes (48, 49). In an alternative pathway, IKKa is activated and phosphorylates p100, stimulating processing of the p52 precursor (50–52).

We and others have reported previously that NF-κB is critical for Tax-mediated p53 inhibition (27, 53). We also demonstrated that the p65/RelA subunit of NF-κB specifically functions in the inhibition of p53 by Tax (28). In this study, we tested whether the IKK complex, which has been shown to regulate p65/RelA (26, 36, 54), plays a role in this pathway. Transient transfections were performed with WT, IKKa−/−, or IKKβ−/− MEFs. We found that Tax failed to inhibit p53 transcriptional activity in IKKβ−/− cells. Overexpression of IKKβ WT, but not KD, rescued Tax-induced p53 inhibition in the IKKβ−/− cells. Further, we showed that treatment with the IKKβ-specific inhibitor SC-514 prevented the inhibition of p53 by Tax. Finally, we investigated whether the phosphorylation of p65/RelA is involved in the regulation of the Tax-induced p53 inhibition pathway. Unlike WT, transfection of the p65/RelA mutant S536A into p65/RelA−/− cells failed to reconstitute Tax inhibition of p53.

It was important to verify whether IKKβ and phosphorylation of p65/RelA at Ser-536 are linked in the pathway to regulate Tax inhibition of p53. Using in vitro kinase assays, we found that C81 cells showed strong IKK kinase activity for both p65/RelA and IκBα. The kinase activity was blocked by SC-514, an IKKβ-specific inhibitor, suggesting that the phosphoryla-
IKKβ and Phosphorylation in Tax Inhibition of p53

Fig. 4. IKKβ induces the phosphorylation of p65/RelA in vitro. A, in vitro IKK kinase assays were performed with HTLV-I-transformed C81 cells. Cells were untreated (lane 1) or treated with SC-514 (lane 2, 50 μM) for 18 h, and cytoplasmic extracts were immunoprecipitated (IP) with anti-IKKβ antibody (Santa Cruz Biotechnology). Kinase reactions were performed in kinase buffer with 1 μg of substrate His6-p65/RelA (left panel) or glutathione S-transferase-IκBα (right panel) at 30 °C for 30 min. Samples were separated by electrophoresis in 4–20% Tris-glycine gel (Novex), and then kinase activity was quantitated on a PhosphorImager. B, GelCode-stained membrane shows that the same amounts of substrate, His6-p65/RelA (left panel) or glutathione S-transferase-IκBα (right panel) were present in each reaction mix. C, Western blot analysis of transfected cells indicates the expression of IKK-β (top panel, Cell Signaling) and phospho-IKK-β (bottom panel, Cell Signaling) levels as visualized by chemiluminescence.

Our studies suggest that a critical step in p53 inhibition is p65/RelA phosphorylation mediated by IKKβ. It is interesting to note that IKK-mediated p65/RelA phosphorylation at Ser-536 has been reported in cancer cell lines including the human cervical carcinoma cell line HeLa and the colorectal adenocarcinoma cell line HT29 (26, 39, 56). The p65/RelA subunit was phosphorylated in vivo. Together, these results strongly suggest that IKKβ phosphates p65/RelA in vitro and in vivo and that IKKβ, but not IKKα, plays a role in the inhibition of p53 by Tax.

O’Mahony et al. (30) reported recently that in Tax transactivation, IKKβ plays a dominant role in signaling for IκBα degradation, whereas IKKα appears to play an important role in enhancing the transcriptional activity of NF-κB by promoting p65/RelA phosphorylation at Ser-536. In these studies, the authors concluded that Tax failed to activate NF-κB reporter constructs in IKKα–/– but not IKKβ–/– MEFs. In our studies, we found that NF-κB luciferase values were 10–20-fold lower in Tax-transfected IKK knock-out cells compared with Tax-transfected WT cells. With this point in mind, however, we did observe a modest increase in NF-κB activity in IKKβ–/– but not IKKα–/– cells. Consistent with our results, others have reported that IKKα–/– and IKKβ–/– cells are deficient in interleukin-1- and TNF-stimulated phosphorylation of the transactivation domain of the p65/RelA subunit and thus, NF-κB transcriptional activity (55).

It is interesting that p53 inhibition requires IKKβ but not IKKα because IKKα and IKKβ share 52% overall sequence homology and are capable of phosphorylating members of the IκB family as well as p65/RelA. In vitro studies indicate that the IKKα/IKKβ heterodimer has higher catalytic activity than either homodimer (57). Moreover, available data indicate that IKKα and IKKβ preferentially form heterodimers in vivo. Nonetheless, knock-out cells show that both IKKα and IKKβ are capable of homodimerization. Of interest to our studies, an IKKβ only complex was shown to exist in HeLa cells by affinity purification, anion exchange chromatography, and immunodepletion analysis, although the complex appeared to be only weakly activated by TNF-α (58). The fact that we did not see a requirement for IKKα argues that IKKβ, either as a homodimer or in complex with IKKγ (NEMO), is required for p53 inhibition through p65/RelA.
We have identified a novel Tax-mediated pathway of p53 inhibition, which involves IKKβ and p65/RelA. IKKβ appears to be directly involved in p65/RelA phosphorylation. These results are surprising in as much as phosphorylation at Ser-536 has been reported to activate the transcriptional potential of p53/RelA by facilitating functional interaction with coactivators such as CBP/p300 (20). It is of interest, therefore, that p65/RelA phosphorylation at Ser-536 contributes to the inhibition of p53 transcriptional activity. Our previous studies have demonstrated that Tax induces the p65/RelA subunit of NF-κB to associate with p53 and stably integrate into the transcriptional complex in vivo (28). This novel inhibition function of p65/RelA may involve inappropriate interaction with coactivators or chromatin-modifying proteins in the transcriptional complex. For example, several studies have indicated that NF-κB-dependent transcription is regulated through interaction of the p65/RelA subunit with histone deacetylase (HDAC) corepressor proteins (59–62). Expression of HDAC1 and HDAC2 repressed TNF-induced NF-κB-dependent transcriptional activity and gene expression (59). It is possible, therefore, that phosphorylated p65/RelA incorporates HDACs into the p53 complex, inhibiting transcriptional activity. Alternatively, the association of p53/p65/RelA at the p53-responsive promoter may inhibit transcription in a manner similar to the p65/RelA inhibition of Vitamin D receptor-mediated transcription. There must be differences in the pathway, however, because Vitamin D receptor inhibition requires the N-terminal Rel homology domain and the mid-molecular phosphoamino acid region that includes Ser-276 (63). p53 inhibition by Tax requires the C-terminal domain and Ser-536 phosphorylation.

Our studies have uncovered a novel role for p56 Ser-36 phosphorylation in mediating p53 transcriptional activity in HTLV-I-infected cells. It will be important to develop strategies to alter this pathway and reactivate p53 and its apoptotic function in transformed cells.

REFERENCES
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