Human T-cell Lymphotropic Virus Type 1 Tax Induction of Biologically ActiveNF-κB Requires IκB Kinase-1-mediated Phosphorylation of RelA/p65

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Activation of the NF-κB/Rel family of transcription factors proceeds through a catalytic complex containing IκB kinase (IKK)-1 and IKK2. Targeted disruption of each of the IKK genes suggests that these two kinases may mediate distinct functions in the activation pathway. In our studies of the human T-cell lymphotropic virus type 1 (HTLV-1) Tax oncoprotein, we have uncovered a new function of IKK1 required for complete activation of the NF-κB transcriptional program. In IKK1−/− murine embryonic fibroblasts (MEFs), Tax normally induced early NF-κB activation events. However, NF-κB induced by Tax in these IKK1−/− cells was functionally impaired. In IKK1−/− (but not wild-type) MEFs, Tax failed to activate several different κB reporter constructs or to induce the endogenous IκBα gene. In contrast, Tax normally activated the cAMP-responsive element-binding protein/activating transcription factor pathway, leading to full activation of an HTLV-1 long terminal repeat reporter construct in IKK1−/− cells. Furthermore, reconstitution of IKK1−/− cells with kinase-proficient (but not kinase-deficient) forms of IKK1 restored the Tax induction of full NF-κB transactivation. We further found that the defect in NF-κB action in IKK1−/− cells correlated with a failure of Tax to induce phosphorylation of the RelA/p65 subunit of NF-κB at Ser529 and Ser536. Such phosphorylation of RelA/p65 was readily detected in wild-type MEFs. Phosphorylation of Ser536 was required for a complete response to Tax expression, whereas phosphorylation of Ser529 appeared to be less critical. Together, these findings highlight distinct roles for the IKK1 and IKK2 kinases in the activation of NF-κB in response to HTLV-1 Tax. IKK2 plays a dominant role in signaling for IκBα degradation, whereas IKK1 appears to play an important role in enhancing the transcriptional activity of NF-κB by promoting RelA/p65 phosphorylation.

Over the past 2 decades, an ever increasing body of work has indicated that the NF-κB/Rel family of transcription factors lies at the crux of such diverse cellular processes as proliferation, differentiation, and death (1–3). Initially identified as pivotal regulators of immune and inflammatory responses, the actions of this family of transcription factors have now expanded to encompass many physiological and pathological conditions, such as fetal development, neuronal function and degeneration, angiogenesis, ischemia, and tumorigenesis (4, 5). Principally found in the cytoplasm under basal conditions, latent NF-κB/IκB complexes are poised to rapidly respond to a variety of external and internal signals, including TNF-α and interleukin-1, and bacterially and virally derived products, such as lipopolysaccharide, HTLV-1 Tax, and UV light (6, 7).

The activation of this NF-κB/Rel family of transcription factors, all of whom share an N-terminal Rel homology domain, is rapid and occurs in the absence of protein synthesis (3). As such, NF-κB must be tightly regulated at multiple levels within the cell, including its release from the inhibitor protein, its mobilization from the cytoplasm into the nucleus, and its engagement of cognate κB enhancers, leading to changes in target gene expression. The activation of NF-κB can be viewed as occurring in two discrete phases. The first phase comprises the proximal events leading to the degradation of the IκB inhibitor and the translocation of the NF-κB complex into the nuclear compartment. This initial phase of NF-κB activation proceeds through a kinase cascade culminating in the site-specific phosphorylation, ubiquitylation, and subsequent degradation of the IκBα inhibitor by the 26 S proteasome. Although the panel of kinases that are recruited after the detection of a particular stimulus includes NIK, TAK-1 (Transforming growth factor-β-activated kinase), and MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1), they all appear to converge at a common point in the pathway, viz. the activation of a large catalytic complex termed the signalosome (8, 9). In turn, the signalosome catalyzes the phosphorylation of the inhibitor protein at two specific N-terminal serines (Ser32 and Ser36 in IκBα and Ser19 and Ser32 in IκBβ) (10–13). This phosphorylation event is mediated by two specific kinases termed IKK1 and IKK2 that reside in the signalosome in conjunction with a third non-catalytic component termed NEMO/IKKγ (14–19). These two highly related kinases appear to act in concert to transmit the activation signal in a directional manner, leading to the phosphorylation of IκBα (20, 21). The second phase in the activation of NF-κB...
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involves the post-translational modification of the NF-κB subunits by both phosphorylation and acetylation (22–31). Although the former phosphorylation events on IκBα are required for release of NF-κB, the latter modifications on RelA/p65 appear to enhance its transcriptional activity and the duration of the response (32). In contrast to the phosphorylation of IκBα, the events that culminate in the modification of RelA/p65 are less well defined. Moreover, the end result appears to vary with respect to cell type, the stimulus evoked, the kinase recruited, and the target residues modified. However, irrespective of these differences, it is clear that the phosphorylation of RelA/p65 augments the transcriptional potential of the NF-κB transcription factor complex. Kinases and their target residues that have been implicated in RelA/p65 modification include protein kinase A (on Ser276) (22, 33), MSK1 (on H9251 reporter construct indicated in the figure legends. After 12 h, TNF-α–induced luciferase activity with both reporter constructs was assayed using a luciferase detection kit (Promega) and a multiwell luminometer (Wallac). Cells were also cotransfected with an expression plasmid encoding a NF-κB–specific DNA binding activity as a signature of NF-κB activation (8). In actuality, complete activation of this transcription factor culminates in its ability to regulate the expression of various target genes. One experimental approach to detect this functional end point is to assess the expression of a reporter gene placed under the control of a NF-κB enhancer element. Accordingly, we first tested the ability of a transiently expressed HTLV-1 Tax protein to induce NF-κB transactivation of the IFN-κB-Luc reporter gene in IKK1−/− MEFs versus WT MEFs. Although Tax induced robust luciferase reporter activity in WT MEFs, we observed only minimal luciferase activity in IKK1−/− MEFs over a range of transfected Tax expression vector DNAs (Fig. 1, A and B). To rule out the possibility that these results reflect a selective failure to activate the IFN-κB enhancer, we tested a second reporter plasmid containing the κB enhancer from the E-selectin gene promoter. A similar defect in Tax induction of luciferase activity was observed with this E-selectin-κB-Luc construct in IKK1-deficient cells expressing Tax (Fig. 1, A and B). Comparable expression of Tax in these IKK1−/− and WT MEFs was confirmed by Western blotting of the lysates with anti-Tax antibodies (Fig. 1E). In contrast to the defect observed with Tax as the agonist, the expression of NIK induced a dose-dependent activation of both κB-Luc reporters. Similarly, stimulation with TNF-α induced luciferase activity with both reporter constructs, although the response observed with IFN-κB was moderately impaired. This functional defect in the Tax response is not attributable to the embryonic stage of development of these IKK1−/− cells because Tax normally activated both of these κB reporters in similarly derived WT MEFs.

Next, we examined the effects of Tax activation of κB-Luc reporters in MEFs lacking IKK2 and in 293 fibroblasts. Loss of IKK2 leads to a reduced, but demonstrable induction of NF-κB-mediated transactivation in response to Tax expression.
FIG. 1. HTLV-1 Tax fails to induce NF-κB transactivation of κB reporter constructs in IKK1-deficient cells. A–D, IKK1−/−, IKK2−/−, and WT MEFs and 293 fibroblasts were transiently transfected with either an IFN-κB-Luc or E-selectin-κB-Luc reporter and Renilla luciferase constructs as indicated. Cells were also transfected with an empty vector or with Tax or NIK expression plasmids (0.05, 0.1, and 0.5 μg/μl) or were stimulated with TNF-α (10 ng/ml). Luciferase activity was detected using an automated 96-well luminometer. NF-κB-induced luciferase levels (relative to control Renilla luciferase levels) obtained in four replicate wells in six independent experiments are shown. Error bars indicate S.E. E, Tax protein expression in each cell type was confirmed by Western blotting. F and G, HeLa cells were transfected using OligofectAMINE with siRNA oligonucleotides corresponding either to a scrambled sequence (si-scr) or to a coding region within the IKK1 open reading frame (si-IKK1). After 24 h, cells were again transfected with the indicated siRNAs together with either an empty vector or a Tax expression vector (0.1 μg/μl) and both κB-Luc and Renilla luciferase reporter constructs. The indicated wells were stimulated with TNF-α after 18 h, and all cells were lysed at 24 h. Luciferase levels were detected as described for A–D. Whole-cell lysates (WCL) were probed by Western blotting with anti-IKK1 antibodies to monitor changes in IKK1 protein levels after siRNA transfection. RLU, relative luciferase units; IB, immunoblot; IP, immunoprecipitation.
Fig. 2. Tax induces HTLV-1 LTR transcription through the CREB pathway, but fails to induce NF-κB-mediated transcription in IKK1−/− MEFs. IKK1−/−, IKK2−/−, and WT MEFs and 293 fibroblasts were transfected with either a 5x-b-Luc plasmid or an HTLV-1-LTR-Luc reporter plasmid. Cells were also transfected with Tax or NIK or were stimulated with TNF-α as indicated. Differences in transfection efficiency were normalized by cotransfection with Renilla luciferase. Note that Tax effectively activated the HTLV-1 LTR (but not the NF-κB enhancer) in IKK1−/− cells. Error bars indicate S.E. RLU, relative luciferase units.

Although the response to Tax was attenuated in IKK2−/− MEFs, we observed greater κB reporter activity in IKK2−/− MEFs than in IKK1−/− cells over a range of Tax expression levels. This induction of NF-κB activity by Tax in IKK2-deficient cells likely involves IKK1-mediated kinase activity, resulting in IκB phosphorylation, degradation, DNA binding, and transactivation by the NF-κB complex (Fig. 1C). In addition, strong Tax responses were observed in 293 fibroblasts (Fig. 1D). The expression of Tax in these cells was confirmed by Western blotting of the lysates with anti-Tax antibodies (Fig. 1E).

To further explore the role of IKK1 in Tax activation of NF-κB, we employed siRNAs to “knock down” the expression of IKK1 in HeLa cells. As shown in Fig. 1 (F and G), transfection of HeLa cells with siRNA specific for IKK1 both inhibited IKK1 protein expression and impaired the induction of b-Luc activity by Tax, but not by TNF-α. Conversely, transfection of these cells with siRNA specific for IKK2 inhibited Tax induction of NF-κB and IKK2 expression, but not that of IKK1 (Fig. 1H–J). These results indicate that Tax activates the NF-κB pathway through IKK1 in these cells.
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FIG. 4. Reconstitution with kinase-proficient (but not kinase-deficient) IKK1 restores Tax-induced activation of biologically active NF-κB. A, IKK1/−/− MEFs were transiently transfected with expression plasmids encoding either kinase-proficient IKK1 or kinase-deficient IKK1 alone or in combination with Tax. Cells were also transfected with both a β-Luc reporter construct and a control Renilla luciferase plasmid. After 24 h, cells were lysed, and the relative amount of luciferase activity was detected using a 96-well luminometer. The results represent four replicate wells in six independent experiments. Error bars indicate S.E. B, WT and IKK1/−/− MEFs were transfected with a kinase-proficient IKK1 expression plasmid alone or with Tax or were stimulated with TNF-α as indicated. After 24 h, cells were lysed, and the amount of IκBα protein resynthesized was quantified by immunoblotting. Cells were stimulated with TNF-α for 10 min to confirm the degradation of IκBα. C, IKK1/−/− MEFs were transfected with kinase-proficient or kinase-deficient IKK1 and Tax. After 24 h, cells were fractionated into cytoplasmic and nuclear compartments. Nuclear lysates were incubated with a radiolabeled κB DNA probe. Protein-DNA complexes were separated by SDS-PAGE and exposed to autoradiography film. RLU, relative luciferase units; KM, K44M.

cells with scrambled siRNA with the same nucleotide composition did not inhibit IKK1 expression or the Tax response.

In addition to the induction of NF-κB, Tax expression also leads to the activation of the CREB/activating transcription factor pathway, which mediates transactivation of the HTLV-1 LTR (39). To determine whether the lack of IKK1 impacts on this Tax signaling pathway, we tested the ability of Tax to induce a CREB-regulated HTLV-1-LTR-Luc reporter in IKK1/−/− cells. In contrast to the functional defect observed in the NF-κB pathway, Tax normally activated the CREB/activating transcription factor pathway, leading to full stimulation of the HTLV-1 LTR reporter gene in IKK1/−/− cells (Fig. 2). Comparable induction of HTLV-1 LTR reporter gene expression was observed in IKK1/−/− and WT MEFs. As expected, the coexpression of NIK or stimulation with TNF-α failed to activate the CREB-regulated reporter construct while inducing a robust κB enhancer-dependent response. Together, these functional studies highlight a key role for IKK1 in Tax-mediated induction of κB-dependent gene expression.

Initial NF-κB Induction Events Are Intact in IKK1/−/− Cells Expressing Tax—To further define this essential role of IKK1 in Tax signaling, we investigated various steps in the NF-κB signaling axis in IKK1/−/− versus WT MEFs and various IKK1-expressing cell lines (Fig. 3A). Of note, Tax functioned normally during the initial stages of NF-κB activation, promoting the activation of IKK (Fig. 3B), degradation of IκBα (Fig. 3C), and nuclear translocation of RelA/p65 (Fig. 3D). These findings underscore the notion that homodimeric IKK2 complexes are fully competent to mediate the phosphorylation events associated with the initial phase of NF-κB activation (Fig. 3B). Tax expression did not induce detectable in vitro phosphorylation of IκBα with NEMO-immunoprecipitated signalosomes from IKK2/−/− MEFs; however, we did observe IκBα degradation in IKK2/−/− MEFs, albeit with slower kinetics than seen in either WT or IKK1/−/− MEFs (Fig. 3C). In contrast, the levels of NF-κB p65 remained unchanged over the same time course in IKK2/−/− MEFs. This decrease in IκBα levels likely results from IKK1-mediated phosphorylation targeting IκBα for proteasome-mediated degradation.

Finally, we did not observe any defect in the binding of NF-κB to a κB DNA probe using nuclear extracts from IKK1-deficient cells expressing Tax (Fig. 3E). Interestingly, in IKK2/−/− MEFs, NF-κB DNA binding was induced in response to Tax expression; however, loss of IKK2 resulted in significantly diminished NF-κB DNA binding in response to TNF-α (Fig. 3E). Together, these results demonstrate that the proximal events in the NF-κB signaling pathway occur normally in IKK1/−/− cells stimulated with HTLV-1 Tax. Nonetheless, NF-κB induced by Tax in these IKK1/−/− cells is functionally impaired as evidenced by its failure to activate several different κB reporter constructs (Fig. 1, A and C).

Reconstitution with Kinase-Deficient (But Not Kinase-Deficient) IKK1 Restores Tax Induction of Biologically Active NF-κB—To determine whether the defect in NF-κB transcriptional activity in IKK1/−/− MEFs induced with Tax was due solely to the absence of IKK1, we reconstituted IKK1/−/− cells with WT IKK1 or kinase-deficient IKK1-K44M vector DNA (Fig. 4A). In WT IKK1-reconstituted cells, the defect in Tax signaling was repaired in the κB reporter assay as well as in the resynthesis of the NF-κB target gene IκBα (Fig. 4B). In contrast, when IKK1/−/− cells were reconstituted with kinase-deficient IKK1, no increase in κB-Luc activity or IκBα resynthesis was observed. Thus, the failure of NF-κB to mediate transcription in response to Tax in IKK1/−/− cells relates specifically to the loss of IKK1 catalytic activity. In addition, reconstitution of IKK1/−/− MEFs with WT (but not kinase-deficient) IKK1 enhanced the binding of NF-κB to its cognate enhancer probe in a gel shift experiment (Fig. 4C).

Recently, IKK1 was shown to be required for the signal-coupled induction of p100 processing to p52, a transactivation partner of RelA/p65 and RelB (40, 41). In IKK1/−/− MEFs, both Tax and NIK fail to induce the proteasome-mediated processing of p100 to p52, rendering the cells deficient in this pathway of NF-κB signaling. To investigate whether this defect could explain the transactivation defect observed in IKK1/−/− cells expressing Tax, we transiently transfected IKK1/−/− cells with an expression plasmid encoding p52. Despite reconstituting p52 levels in these cells, the defect in κB reporter transactivation in response to Tax was not repaired, suggesting that IKK1 mediates an additional role required for Tax induction of NF-κB action (Fig. 5A). Furthermore, examination of the nuclear complexes induced in WT MEFs expressing Tax revealed
that the major species of NF-κB elicited contained p50 and RelA/p65. Thus, failure to induce p52 processing does not appear to explain the transactivation defect observed in IKKI−/− MEFs stimulated with Tax (Fig. 5B).

**Tax Fails to Induce RelA/p65 Phosphorylation in IKKI-deficient MEFs**—The observation that IKKI kinase activity was required for full activation of NF-κB action by Tax supports the notion that the phosphorylation of a substrate is a key component of this response. Recently, several groups identified IKK2 as a kinase capable of phosphorylating the RelA/p65 subunit of the NF-κB complex (22, 23, 26–29, 33). Because we observed a defect in Tax signaling in IKKI−/− cells, the ability of Tax to induce the phosphorylation of the RelA/p65 subunit was assessed in an *in vitro* kinase assay. NEMO-containing complexes were immunoprecipitated from IKKI−/− MEFs, WT MEFs, 293 cells, or IKK2−/− MEFs transfected with Tax or stimulated with TNF-α. In both WT MEFs and 293 cells, we observed that Tax and TNF-α induced robust phosphorylation of a full-length RelA/p65 substrate in *vitro*. In addition, using immunoprecipitated IKK1–NEMO complexes, we observed that both Tax and TNF-α induced p65 phosphorylation in IKK2−/− MEFs. In contrast, although TNF-α induced RelA/p65 phosphorylation in IKKI-deficient MEFs, Tax did not (Fig. 6A). Of note, reconstitution of IKKI−/− MEFs with kinase-proficient (but not kinase-deficient) IKK2 restored the phosphorylation of RelA/p65 in Tax-activated cells (Fig. 6B). Thus, although IKK2 is capable of phosphorylating RelA/p65 in *vitro*, this activity is not sufficient to support the Tax-mediated response in IKKI−/− cells. With respect to TNF-α stimulation, we did not observe a defect in either IKKI−/− or WT MEFs, suggesting the involvement of kinase(s) other than IKK1 as catalytic responders to TNF-α. This finding is in agreement with other reports implicating IKK2, casein kinase II, and MSK1 as mediators of RelA/p65 phosphorylation (22, 23, 26, 27, 33, 34).

To map the phosphorylation site(s) of RelA/p65 targeted by Tax stimulation of IKKI, we used constructs encoding either the N terminus (p65ΔN(1–305) or C terminus (p65ΔN(354–551)) of RelA/p65 as substrates in an *in vitro* kinase assay. In Tax-activated WT MEFs, we observed that the vast majority of phosphorylation occurred in the C-terminal region of the protein, with little or no Tax-induced phosphorylation detected in the N terminus (Fig. 6C). TNF-α induced phosphorylation in the both N- and C-terminal regions of the RelA/p65 protein, suggesting that this stimulus induces the phosphorylation at a number of sites within RelA/p65 perhaps by recruiting a range of kinases. Other studies have implicated serines within this C-terminal region as targets of phosphorylation, viz. Ser252 and Ser256. We generated a baculovirus-expressed, His-tagged mutant of full-length RelA/p65 in which Ser252 was mutated to alanine. Using NEMO-immunoprecipitated complexes from either WT or IKK1−/− MEFs, we observed that, although phosphorylation of the S536A mutant was reduced in response to Tax in WT MEFs, it was not abolished. As expected, we failed to see any Tax-induced phosphorylation of RelA/p65 or its mutant in IKKI−/− MEFs (Fig. 6D). These data suggest that Tax, acting through IKKI1, targets an additional residue(s) within the C terminus of RelA/p65. Using GST fusion constructs of the C terminus in which Ser252 and Ser256 were substituted with alanines either individually or in combination (p65ΔN-S529A, p65ΔN-S536A, and p65ΔN-S529A/S536A), we observed that both residues were targeted for phosphorylation by Tax-activated complexes from WT MEFs (Fig. 6E). When one or the other individual serine mutant was used as a substrate, the level of RelA/p65 phosphorylation was decreased, but was not eliminated. However, when both serines were mutated to alanines, a complete loss of Tax-induced phosphorylation of the C terminus of RelA/p65 occurred. Interestingly, upon NIK expression or TNF-α stimulation, we observed residual phosphorylation of the C terminus of the RelA/p65 double mutant, suggesting that alternative residues are targeted in response to these stimuli. To correlate the Tax-induced phosphorylation of p65 with its transcriptional activity, we assessed the induction of NF-κB-Luc in p65−/− MEFs reconstituted with either WT p65 or various phosphorylation site mutants (p65ΔN-S529A, p65ΔN-S536A, and p65ΔN-S529A/S536A) (Fig. 6F). Tax induced a robust response in p65−/− MEFs reconstituted with WT p65 over a range of Tax expression levels. Similarly, TNF-α also induced a robust response in these WT p65-reconstituted MEFs. Conversely, mutation of both phosphorylation sites (Ser252 and Ser256) resulted in a nearly complete loss of reporter gene activity. Reconstitution of p65−/− MEFs with expression vectors encoding p65 mutated at the individual phosphorylation sites resulted in different levels of reporter gene activity. Although mutation of Ser256 in p65 produced a significant reduction in the amount of luciferase activity induced by Tax (70% inhibition), the decrease in reporter activity was much less in p65−/− MEFs reconstituted with the p65ΔN-S529A expression vector (20% inhibition). Although Tax does induce p65 Ser252 phosphorylation, its role in NF-κB-mediated transactivation appears to be less important. In contrast, the phosphorylation of p65 at Ser256 plays a vital role in the Tax induction of NF-κB transcriptional activity. Together, these findings indicate that Tax activation of IKKI1 leads to the phosphorylation of RelA/p65 specifically at Ser252 and Ser256. IKKI thus plays a key role in the Tax-induced NF-κB response promoting the full biological activity of RelA/p65.

**DISCUSSION**

HTLV-1-mediated transformation of human T-cells underlies development of adult human T-cell leukemia (42). Based on a number of studies, the 40-kDa Tax protein corresponds to the key oncogenic protein produced by this pathogenic human retrovirus. In addition to its ability to enhance the expression of viral genes within the infected cell through transactivation of the HTLV-1 LTR, Tax also functions as a powerful transactivator of select cellular genes that likely contribute to T-cell transformation (43). Tax does not directly bind DNA, but rather acts by usurping normal cellular processes regulating transcription. One such target of Tax corruption is the signal-
ing pathway that leads to the activation of NF-κB. Many of the target genes of NF-κB function in immune cell proliferation as well as in protection from apoptotic cell death; thus, this transcription factor forms a potentially important target for deregulation by HTLV-1 Tax.

Targeted disruption of each of the IKK genes has provided further insight into their critical, but distinct functions (44). IKK2 corresponds to the principal kinase catalyzing the phosphorylation of IκB in the initial phase of NF-κB activation (45–47).

FIG. 6. Tax fails to induce required RelA/p65 phosphorylation in IKK1-deficient MEFs. A, WT, IKK1−/−, and IKK2−/− MEFs and 293 cells were transfected overnight with Tax or were stimulated with TNF-α (10 ng/μl) for 10 min. Lysates were incubated for 2 h with anti-NEMO antibodies or with both anti-NEMO and anti-IKK1 antibodies (IKK2−/− MEF lysates) and protein A-agarose to immunoprecipitate the IKK complexes. Immunoprecipitated complexes were subjected to an in vitro kinase assay (IVKA) using a baculovirus-purified, His-tagged p65 substrate (1 μg/ml) in the presence of [γ-32P]ATP. Reaction products were separated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, followed by autoradiography. The expression of Tax in all lysates was confirmed by immunoblotting (IB) with Tax-specific antiserum. B, WT and IKK1−/− MEFs were transfected with Tax or were stimulated with TNF-α. IKK1−/− MEFs were also transfected with either WT IKK1 or IKK1-K44M plasmids as indicated and incubated overnight. NEMO-containing complexes were immunoprecipitated and subjected to an in vitro kinase assay as described for A. C–E, WT MEFs were transfected with either Tax or NIK as indicated and incubated overnight. Alternatively, cells were stimulated with TNF-α for 10 min. NEMO-containing IKK complexes were immunoprecipitated from whole-cell lysates as described. Immunoprecipitated complexes were subjected to an in vitro kinase assay using various p65 substrates, including His-tagged full-length p65, GST-tagged p65 constructs corresponding to either the N terminus (residues 1–305) or C terminus (residues 354–551) of p65, and GST-tagged p65 constructs corresponding to C-terminal residues 334–551 and mutated at either or both Ser529 and Ser536. Radiolabeled products were detected by SDS-PAGE and autoradiography. F, p65−/− MEFs reconstituted with either WT p65 or various phosphorylation site mutants (p65-S529A, p65-S536A, and p65-S529A/S536A) were transiently transfected with IκB-Luc reporter and Renilla luciferase constructs. Cells were also transfected with an empty vector or Tax (0.1, 0.4, and 0.8 μg/ml) or were stimulated with TNF-α (10 ng/ml). Luciferase activity was detected using an automated 96-well luminometer. Luciferase levels were obtained from four replicate wells in three independent experiments. Error bars indicate S.E. RLU, relative luciferase units.
subtler with respect to NF-κB activation by such inducers as TNF-α and interleukin-1, perhaps reflecting compensatory actions of IKK2 homodimers (48–50). However, when HTLV-1 Tax is employed as the inducer, clear functional differences emerge with respect to the roles these kinases play in the induction of NF-κB activity. As an inducer, Tax has been well characterized with respect to the activation of early events in the NF-κB signaling pathway (39, 51, 52). For example, Tax has been shown to bind to and activate the IKK signalsome via interaction with NEMO (53, 54). Previous studies have also shown that the expression of Tax leads to sustained phosphorylation and turnover of IκBα (37). Of note is the observation that the levels of IκBα mRNA are increased ~7- to 20-fold in Tax-expressing cells. This suggests that, in addition to the sustained activation of IKK kinase activity, Tax leads to the production of fully active NF-κB complexes. We now demonstrate that the induction of such functional NF-κB complexes is critically dependent on the enzymatic function of IKK1.

We have previously reported that that the catalytic activity of IKK1 mediates a key regulatory influence on the kinase activity of the heterodimeric IKK1-IKK2 signalsome in response to various stimuli (20). More recently, IKK1 has been shown to orchestrate a nucleosomal function with respect to gene transcription by activated NF-κB (55, 56). Specifically, these studies have shown that IKK1 is recruited to the promoter region of TNF-α-induced target genes, where it phosphorylates Ser10 in histone 3 and enhances NF-κB-mediated transactivation (55, 56). In this study, we further expanded the role of IKK1 in modulating the activation of NF-κB by Tax, showing that this kinase induces the phosphorylation of the C terminus of RelA/p65, which is key for full biological activity of the induced NF-κB complex. Although proximal signaling events leading to NF-κB nuclear translocation and DNA binding occur normally in Tax-stimulated cells lacking IKK1, the biological activity of these NF-κB complexes is greatly diminished. In IKK1−/− MEFs, we failed to detect transactivation of several κB reporters and resynthesis of the target gene IκBα. This defect in the Tax response in IKK1-null cells is specific to the NF-κB pathway because full transactivation of the CREB/activating transcription factor pathway is preserved in response to Tax.

One possible explanation for this defect is provided by the recent observation that Tax fails to induce the processing of p100 to p52 (a partner of RelA/p65) in IKK1−/− cells (41). In our study, however, reconstitution of IKK1−/− cells with exogenous p52 failed to rescue the transactivation defect. Moreover, examination of nuclear extracts revealed that p50-RelA/p65 complexes were the principal NF-κB species induced by Tax in WT MEFs.

To identify where the defect in Tax activation of transcriptionally active NF-κB complexes lies, we confirmed that the kinase activity of IKK1 is required for both the initial and the secondary phases of this activation pathway. This finding suggests that, downstream of IκBα degradation, a key intermediate is phosphorylated by Tax-activated IKK1. One possible target is RelA/p65 itself, as this subunit serves as a substrate for several kinases, including protein kinase A, casein kinase II, MSK1, and IKK2 (22, 23, 26–29, 33, 57). However, IκBα phosphorylation appears to be conserved in response to a wide variety of stimuli, the phosphorylation of RelA/p65 appears to differ with respect to the stimulus, the cell type, and the target residue of RelA/p65. For example, lipopolysaccharide stimulation of cells leads to protein kinase A-mediated phosphorylation of Ser320 in RelA/p65, leading, in turn, to enhanced association of phospho-RelA/p65 with CREB-binding protein (22, 33). Further support for the importance of this site in NF-κB action stems from experiments in which reconstitution of RelA/p65−/− cells with a RelA/p65-S276A mutant failed to rescue a defect in interleukin-6 production or protect against apoptosis in response to TNF-α or interleukin-1 (28, 29). More recently, this site has been shown to be targeted by the MSK1 kinase activated by both extracellular signal-regulated kinase and p38 during the TNF-α response (28, 29). However, this position is constitutively phosphorylated in endothelial cells and does not appear to affect NF-κB action in this cellular setting (58). Additional residues within the transactivation domains have also been shown to be important for NF-κB action. Specifically, Ser529 has been shown to be phosphorylated by casein kinase II in response to TNF-α signaling (27), whereas IKK2 has been shown to phosphorylate Ser536 (23). We have confirmed that Tax activation induces the phosphorylation of RelA/p65 at two C-terminal residues (Ser529 and Ser536) in the transactivation domain of RelA/p65. Although Tax induces the phosphorylation of both of these residues, functionally, it appears that the phosphorylation of Ser536 is critical for the transactivation response. In contrast, the phosphorylation of Ser529 appears to be less critical for the induction of NF-κB-mediated transactivation, although it does appear to contribute to achieving the maximal response to Tax expression. We observed that p65 phosphorylation occurred in the absence of IKK2, but not IKK1, indicating that Tax proceeds through IKK1 to phosphorylate one or both of these serine residues. These data explain why IKK2 homodimers cannot fully compensate for the loss of IKK1 in mediating the response to Tax. We have confirmed that this modification of RelA/p65 by IKK1 in response to Tax expression directly influences the transactivation potential of the RelA/p65-containing complex. One possible mechanism for these effects may involve subsequent modifications of RelA/p65, including acetylation (31), which has been shown to potentiate the transcriptional action of NF-κB.

Together, these findings are consistent with distinct roles for the IKK1 and IKK2 kinases in the activation of NF-κB by Tax. Our studies expand the role of IKK1 with respect to Tax-mediated activation of NF-κB. IKK1 appears to play an important role in enhancing the transactivation function of NF-κB in response to the expression of Tax, whereas IKK2 plays a dominant role in signaling for IκBα degradation during the initial phase of NF-κB activation. Such segregation of function between IKK1 and IKK2 highlights an intriguing genetic economy within the signalsome complex.

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