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Activation of the IκB kinase complex by HTLV-1 Tax requires cytosolic factors involved in Tax-induced polyubiquitination

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Activation of NF-κB by human T cell leukaemia virus type 1 Tax is thought to be crucial in T-cell transformation and the onset of adult T cell leukaemia. Tax activates NF-κB through activation of the IκB kinase (IKK) complex, similar to cytokine-induced NF-κB activation, which involves active signalling complex formation using polyubiquitin chains as a platform. Although polyubiquitination of Tax was reported to be required for IKK activation, most studies have been performed using intact cells, in which secondary NF-κB activation can be induced by various cytokines that are secreted due to Tax-mediated primary NF-κB activation. Therefore, a cell-free assay system, in which IKK can be activated by adding highly purified recombinant Tax to cytosolic extract, was used to analyse Tax-induced IKK activation. In contrast to the cytosolic extract, the purified IKK complex was not activated by Tax, whereas, it was efficiently activated by MEKK1, that does not require polyubiquitination to activate IKK. Moreover, Tax-induced IKK activation was blocked when the cytosolic extract was mixed with either lysine-free, methylated or K63R ubiquitin. These results obtained through our cell-free assay suggest that K63-linked polyubiquitination is critical, but linear polyubiquitination is dispensable or insufficient for Tax-induced IKK activation.

Keywords: Human T cell leukaemia virus type 1/IκB kinase/NF-κB/polyubiquitination/Tax.

Abbreviations: ATL, Adult T-cell leukaemia; CYLD, cylindromatosis; HTLV-1, Human T cell leukaemia virus type 1; IKK, IκB kinase; MEKK1, MAPK/ERK kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; TRAF6, TNF receptor-associated factor 6.

Human T-cell leukaemia virus type 1 (HTLV-1) infects and transforms $CD4^+$ T cells *in vitro* and is etiologically associated with an acute T cell malignancy, Adult T-cell leukaemia (ATL) (*1*). The HTLV-1 genome encodes the Tax protein, which plays a critical role in T-cell transformation (*2*, *3*). Tax regulates the expression of cellular genes involved in T-cell proliferation, cell survival and anti-apoptosis by modulating various transcription factors, including nuclear factor-κB (NF-κB), cAMP-responsible element binding protein (CREB) and serum response factor (SRF) (*1*, *4*). NF-κB is one of the key transcription factors that facilitate cell transformation because the Tax mutant M22, which can activate CREB but not NF-κB, is unable to immortalize T cells (*5*). Therefore, elucidation of the mechanism that underlies Tax-induced NF-κB activation may aid in preventing the onset of ATL.

NF-κB is normally sequestered in the cytoplasm by associating with inhibitory proteins from the NF-κB family (IκBs). Extracellular stimuli, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1, activate the IκB kinase (IKK) complex, that is composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit NF-κB essential modulator (NEMO). The activated IKK complex phosphorylates IκBα, which, in turn, induces Lys48-linked polyubiquitination and proteasomal degradation of IκBα. NF-κB is then translocated into the nucleus and promotes transcription of its target genes (*6*). It has been well established that Lys63-linked polyubiquitination is involved in the cytokine-mediated NF-κB activation pathway (*7*). In the interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) signalling pathways, TNF receptor (TNFR)-associated factor 6 (TRAF6), an E3 ubiquitin ligase, conjugates Lys63-linked polyubiquitination to itself and TGF-β-activated kinase (TAK) 1 together with Ubc13, an E2 ubiquitin-conjugating enzyme (*8*, *9*). Polyubiquitination leads to formation of a signalling complex that contains MAPK/ERK kinase (MEKK3), TAK1, TAK1-binding (TAB) 2/TAB3 and the IKK complex, which induces activation of TAK1 and the IKK complex (*10*). In TNFR1 signalling, cIAPs act as an E3 ubiquitin ligase that conjugates polyubiquitin chains to receptor-interacting protein (RIP) 1 (*11*, *12*). Polyubiquitin chains that are conjugated to RIP1 also act as platforms for the formation of a signalling complex to activate downstream molecules.

To date, many studies have been conducted to elucidate the mechanism of Tax-induced IKK activation. Tax binds to NEMO and induces activation of the IKK complex (*13*, *14*). We have shown that, unlike the cytokine-mediated NF-κB signalling pathway, Tax does not require Ubc13 and MAP3Ks, including TAK1, MEKK1, MEKK3, NF-κB-inducing kinase (NIK) and tumor progression locus (TPL)-2 for IKK

activation (*15*). We have further shown that expression of cylindromatosis (CYLD) does not affect Tax-induced NF-κB activation, whereas it does inhibit TRAF6-induced NF-κB activation (*15*), which led us to hypothesize that polyubiquitination may not be required for Tax-induced NF-κB activation. However, other groups have shown that Ubc13 and TAK1 are required for Tax-induced NF-kB activation (*16*). Therefore, the molecular mechanisms for Tax-induced IKK activation are controversial and largely unknown.

Herein, we establish a cell-free assay system that induces IKK activation in response to recombinant Tax and demonstrate that Tax requires unidentified cytosolic factor(s) to activate the purified IKK complex. We further show that a ubiquitin mutant, which is unable to form polyubiquitin chains, inhibit activation of the IKK complex, which suggests that polyubiquitination is involved in the Tax-induced IKK activation pathway.

Experimental Procedures

Plasmids

The cDNAs that encode Tax, M22, TRAF6, MEKK1 (the C-terminal 321 amino acids) were inserted into the pFastBac™ HT A vector (Invitrogen) to generate a recombinant baculovirus for expression of these proteins in Sf9 cells.

Cell culture and reagents

The Jurkat and JM4.5.2 cells were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS). The Sf9 cells were maintained in Sf900™ II SFM (Gibco) with 10% heat-inactivated FBS. Wild-type (WT) and *Traf6*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (*17*) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FBS. BAY 11-7082 and BMS-345541 were obtained from Calbiochem. The wild-type ubiquitin and ubiquitin mutants were purchased from Boston Biochem.

Purification of the recombinant proteins

His_6-tagged Tax, M22, TRAF6 and MEKK1 were produced using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Briefly, a 50-ml culture of Sf9 cells (1×10^6 cells/ml) was infected with a recombinant baculovirus that expressed each protein. On completing 72 h after infection, the cells were harvested, and the His_6-tagged recombinant proteins were purified using the Ni-NTA resin (QIAGEN) according to the manufacturer's instructions.

Preparation of cytosolic extract (S100) from Jurkat cells

The Jurkat cells (1.5×10^8 cells) were resuspended in 500 µl of hypotonic buffer [10 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and a protease inhibitor cocktail (Roche)] and homogenized using a Dounce homogenizer. The cell debris was removed by ultracentrifugation at 100,000*g* for 1 h, and the cleared supernatant (S100) was collected.

Cell-free assay for IKK activation

The Jurkat cytosolic extract (10 mg/ml) was incubated with the indicated amount of either recombinant Tax, M22, TRAF6 or MEKK1 in ATP buffer [50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 5 mM NaF, 20 mM β-glycerophosphate, 1 mM Na_3VO_4 and a protease inhibitor cocktail]. After incubation at 30°C for 1 h, the reaction mixtures were immunoblotted, immunoprecipitated or pulled down. For the cell-free assay using the purified IKK complex, the IKK complex was immunoprecipitated with an anti-Flag antibody from Jurkat cells that stably expressed Flag-NEMO (5.0×10^8 cells). The purified IKK complex was incubated with the indicated recombinant proteins in the presence of 100 ng of GST-IκBα (amino acids 1–54) in ATP buffer.

Immunoblotting

Either the immunoprecipitates or the whole-cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore). The membranes were incubated with the appropriate primary antibodies. Immunoreactive proteins were visualized with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences), followed by processing with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The following antibodies were used: anti-p-IκBα (9246), anti-IκBα (9242), anti-p-IKKα/IKKβ (2681), anti-IKKα (2682), anti-IKKβ (2684) and anti-NEMO (2695) from Cell Signaling Technology as well as ubiquitin (sc-8017) (Santa Cruz Biotechnology), anti-Tubulin (CP06) (Calbiochem), anti-His-tag (PM032) (MBL) and anti-Tax (Lt-4).

Pull-down assay and immunoprecipitation

For the pull-down assays, pull down buffer [10 mM Tris–HCl [pH 7.0], 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 10 mM imidazole] and Ni-NTA resin were added to an *in vitro* assay reaction mixture. After incubation at 4°C for 1 h, the Ni-NTA resin was washed three times with the pull-down buffer and immunoblotted. To detect polyubiquitination of Tax and NEMO, the reaction mixtures were boiled for 10 min with 1% SDS to remove non-covalently attached proteins. The reaction mixtures were diluted 10-fold in Tris/NaCl/EDTA (TNE) buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 5 mM *N*-ethylmaleimide and a protease inhibitor cocktail] to reduce the concentration of SDS to 0.1%. The reaction mixtures were then incubated with antibodies against Tax or NEMO. The immunoprecipitates were washed three times with TNE buffer and immunoblotted using an anti-Ub antibody.

Results and Discussion

Establishment of a cell-free assay system that reproduces Tax-induced IKK complex activation

Once Tax activates NF-κB, various cytokines, including TNF-α and IL-1, may be induced by NF-κB and secreted, which in turn further activates NF-κB through the cytokine-specific receptors on the Tax-expressing cells (*18*, *19*). This potential autocrine-stimulated NF-κB activation enhances the difficulty of distinguishing signalling events induced by Tax from those induced by cytokines. Therefore, we tried to establish an *in vitro* cell-free system, in which Tax-induced IKK activation can be analysed without cytokine-induced IKK activation. The involvement of polyubiquitination in Tax-induced IKK activation is unclear, although its involvement in cytokine-induced IKK activation has been established (*7*). Thus, the cell-free system is suitable to address the role of polyubiquitin chains in Tax-induced IKK activation. We first expressed recombinant His_6-Tax and the Tax mutant M22, which is defective in NF-κB activation, in Sf9 cells and purified them using Ni-NTA agarose (Fig. 1A) To examine whether recombinant Tax activates the IKK complex, the cytosolic extract was prepared from the human T cell line Jurkat and incubated with either recombinant Tax or M22, and the resulting reaction mixture was analysed using immunoblots with anti-p-IκBα and anti-p-IKKα/β antibodies. Recombinant Tax, but not recombinant M22, induced phosphorylation of IκBα in a dose-dependent manner (Fig. 1B). Phosphorylation of IκBα was also indicated by the appearance of a slower migrating form of IκBα (Fig. 1B). Phosphorylation of IKKα/β was also

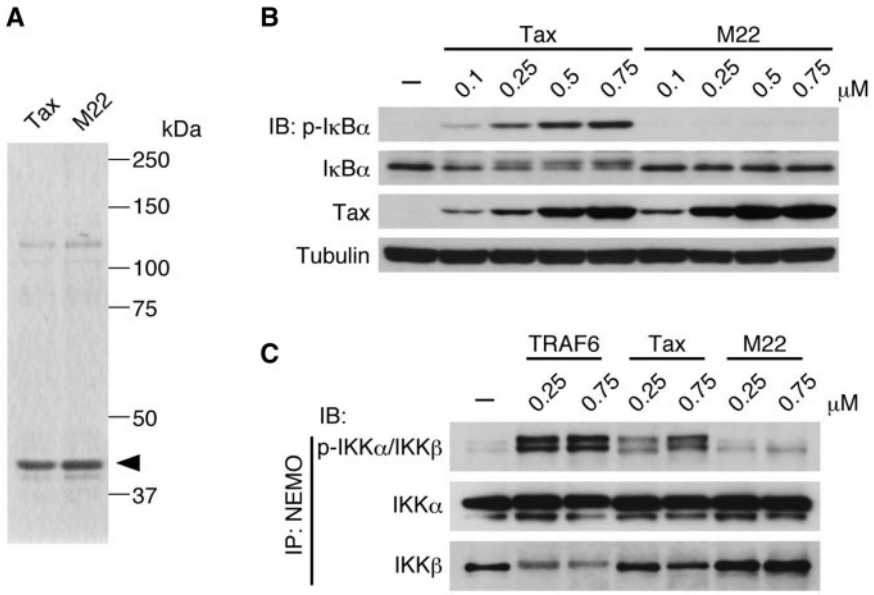

Fig. 1 Recombinant Tax activates the IKK complex in the cell-free assay system. (A) Purification of recombinant His_6-Tax and the Tax M22 mutant. We performed SDS–PAGE on the recombinant Tax and M22 that were purified from Sf9 cells, which was followed by silver staining. An arrow indicates the Tax protein. (B) Recombinant Tax induces phosphorylation of IκBα in a dose-dependent manner. The Jurkat cytosolic extract (10 mg/ml) was incubated with the indicated amount of recombinant either Tax or M22 with ATP (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with the anti-p-IκBα, anti-IκBα, anti-Tax and anti-Tubulin antibodies. (C) Recombinant Tax induces phosphorylation of the IKK complex. The Jurkat cytosolic extract (10 mg/ml) was incubated with the indicated amount of recombinant TRAF6, Tax or M22 at 30°C for 1 h. The reaction mixtures were immunoprecipitated with an anti-NEMO antibody and then immunoblotted with anti-p-IKKα/IKKβ, anti-IKKα and anti-IKKβ antibodies.

induced by recombinant Tax, but not by M22, to a similar extent as induced by TRAF6 (Fig. 1C).

To further confirm that recombinant Tax-induced phosphorylation of IκBα is catalysed by IKK, two distinct IKK inhibitors, BAY 11-7082 (*20*) and BMS-345541 (*21*), were added to the cell-free IKK activation system. Both of the IKK inhibitors inhibited Tax-induced phosphorylation of IκBα in a dose-dependent manner (Fig. 2A and B). Moreover, we performed the cell-free assay using cytosolic extract prepared from JM4.5.2, which is a NEMO-deficient Jurkat cell line, because the interaction of Tax with NEMO is indispensable to Tax-induced IKK activation (*22*, *23*). We confirmed that NEMO was not expressed in JM4.5.2 cells, whereas, IKKα and IKKβ were expressed to a similar extent in the JM4.5.2 and Jurkat cells (Fig. 2C, left). Previous study has shown that TRAF6 fails to induce IκBα phosphorylation in the absence of NEMO (*9*). While we reproduced the TRAF6 requirement for NEMO, recombinant Tax also failed to induce IκBα phosphorylation in the NEMO-deficient cytosolic extract (Fig. 2C, right). To examine whether Tax binding to the IKK complex depends on NEMO in the cell-free assay, Tax was pulled down with Ni-NTA beads, and IKKα/β binding to Tax was analysed by immunoblots. Recombinant Tax interacted with the IKK complex in the Jurkat cytosolic extract, whereas, Tax did not bind the IKK complex without NEMO (Fig. 2D). Taken together, Tax binds the IKK complex through NEMO and subsequently activates the IKK complex in the cell-free assay system. Given that M22, which is unable to bind NEMO (*14*), does not activate the IKK complex in the cell-free system (Fig. 1B and C), these results strongly suggest that our cell-free assay system reconstitutes the Tax-mediated NF-κB activation in intact cells. A similar assay system has been reported previously using partially purified recombinant Tax generated in *Escherichia coli* (*24*). As the purity of our Tax protein generated in sf9 cells is >95% (Fig. 1A) and its specific activity may be >8-fold over that of the Tax from *E. coli* [a minimum concentration of the Tax from Sf9 required for IKK activation is ~0.1 μM (Fig. 1B) while that of the Tax from *E. coli* is ~0.8 μM (*24*)], our system is well suited for further investigation of the molecular mechanism for Tax activation of NF-κB.

Tax requires cytosolic intermediary factors for activation of the purified IKK complex

Using the cell-free systems, we next addressed whether Tax alone induces IKK activation. The IKK complex was purified from unstimulated Jurkat cells and then incubated with either recombinant Tax or other NF-κB activators and recombinant GST-IκBα. As MEKK1 alone is known to activate the purified IKK complex without the polyubiquitination reaction (*9*, *25*), we used MEKK1 as a positive control. On the other hand, TRAF6 requires other molecules, such as Ubc13, Uev1a, TAK1 and TAB2, for IKK activation (*9*, *26*). Therefore, TRAF6 was used as a negative control. When incubated with Jurkat cytosolic extracts, all the recombinant proteins were able to induce phosphorylation of IκBα (Fig. 3A). However, when the recombinant proteins were incubated with the purified IKK complex, only MEKK1 induced phosphorylation of IκBα and IKKβ (Fig. 3B, +IKK).

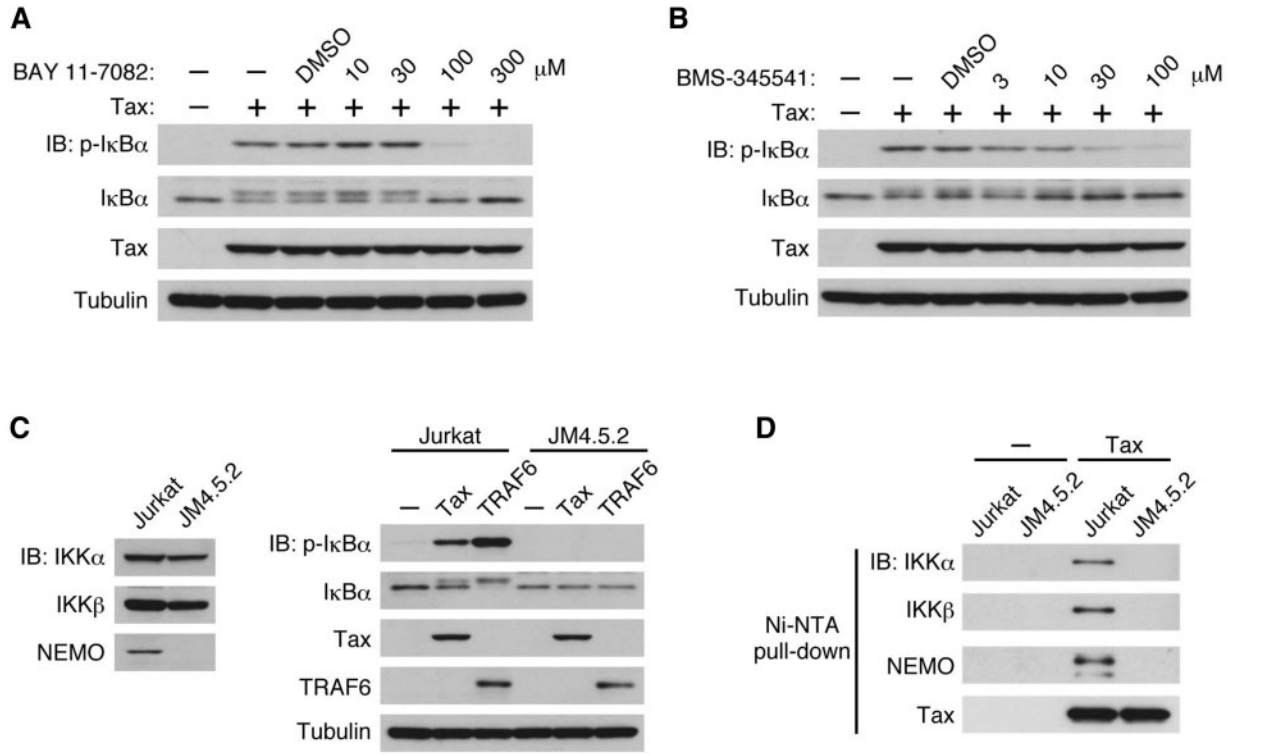

Fig. 2 Phosphorylation of IκBα induced by recombinant Tax is dependent on IKK activation. (A and B) IKK inhibitors inhibit IκBα phosphorylation induced by recombinant Tax in a dose-dependent manner. The Jurkat cytosolic extract (10 mg/ml) was incubated with recombinant Tax (0.5 μM) and ATP (2 mM) and either the IKK inhibitor BAY 11-7082 (A) or BMS-345541 (B) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody. (C) NEMO is essential for Tax-induced phosphorylation of IκBα. The expression levels of IKKα, IKKβ and NEMO were analysed by immunoblot (left). Either the Jurkat or JM4.5.2 cytosolic extract (10 mg/ml) was incubated with either recombinant TRAF6 (0.5 μM) or Tax (0.5 μM) and ATP (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody (right). (D) Tax binding to the IKK complex depends on NEMO. The cell-free assay was performed as described in (C), and the reaction mixtures were then subjected to a Ni-NTA pull-down assay to analyse interaction of Tax with the IKK complex subunits by immunoblot with anti-IKKα, anti-IKKβ and anti-NEMO antibodies.

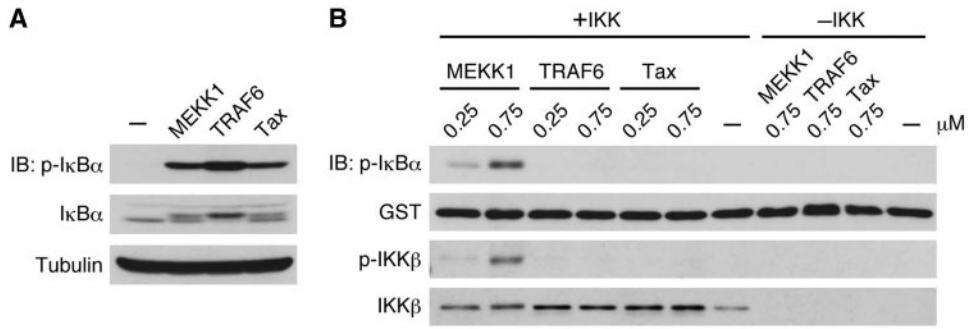

Fig. 3 Tax requires intermediary factors for IKK activation. (A) Recombinant MEKK1, TRAF6 and Tax activated the IKK complex in the Jurkat cytosolic extract. The Jurkat cytosolic extract (10 mg/ml) was incubated with either recombinant MEKK1 (0.75 μM), TRAF6 (0.75 μM) or Tax (0.75 μM) and ATP (2 mM) at 30°C for 1 h. Phosphorylated IκBα was detected by immunoblot with an anti-p-IκBα antibody. (B) Recombinant Tax did not activate the purified IKK complex. Recombinant MEKK1, TRAF6 or Tax was incubated with ATP (2 mM) and GST-IκBα (100 ng) at 30°C for 1 h either without (−IKK) or with (+IKK) the IKK complex purified from unstimulated Jurkat cells. The reaction mixtures were analysed by immunoblot with anti-p-IκBα, anti-GST, anti-p-IKKβ and anti-IKKβ antibodies.

MEKK1 was unable to induce phosphorylation of IκBα without the IKK complex (Fig. 3B, −IKK), indicating that MEKK1 does not directly phosphorylate IκBα. Similar to TRAF6, Tax alone did not activate the purified IKK complex (Fig. 3B), indicating that Tax requires intermediary factors for IKK activation, which are in the Jurkat cell cytosol.

Polyubiquitination is involved in Tax-induced IKK activation but not in the interaction between Tax and NEMO

We next sought to determine that cytosolic intermediary factor(s) required for Tax-induced IKK activation. It has been well established that various polyubiquitin chains are crucial in IL-1- and TNF-α-induced IKK

activation (*7*). IL-1 induces the polyubiquitinated forms of TRAF6 and TAK1, and TNF-α induces those of RIP1 (*8*, *9*, *11*, *12*). Moreover, it has been reported that unanchored polyubiquitin chains are generated in response to these stimulating events (*27*). These polyubiquitin chains act as a platform for the formation of an active TAK1-containing signalling complex, which activates IKK. In addition to TAK1 activation, the activation of IKK requires stimulation-induced Lys63-linked or linear polyubiquitin chain conjugation to NEMO (*28–32*), which may induce either oligomer formation or a conformational change in NEMO to activate the IKK complex. The former conjugation is catalysed by TRAF6 with Ubc13 and the latter by the linear ubiquitin chain assembly complex (LUBAC), which is composed of HOIL-1, HOIP and Sharpin (*33–35*). We have previously shown that Ubc13 is dispensable to Tax-induced IKK activation, and expression of CYLD does not affect Tax-induced NF-κB activation (*15*). However, we cannot exclude the possibility that Lys63-linked polyubiquitination is involved in Tax-induced IKK activation because E2 ubiquitin-conjugating enzymes other than Ubc13 could be involved and that the Lys63-linked polyubiquitin chains could be somehow blocked from CYLD-mediated deubiquitination. Because our cell-free assay system is not affected by the Tax-induced secretion of various cytokines that trigger generation of polyubiquitin chains, we next investigated whether polyubiquitination was required in Tax-mediated IKK activation using the cell-free system. To inhibit polyubiquitin chain formation, we used two ubiquitin mutants. We used a lysine-free ubiquitin mutant, in which all the lysine residues were mutated to arginine (K0-Ub). We also used a methylated ubiquitin, in which the amino groups, including the ε-amino group, on all the lysine residues and the N-terminal Met α-amino group were blocked by methylation (Me-Ub). The addition of these ubiquitin mutants suppressed TRAF6-induced IKK activation, whereas MEKK1-induced IKK activation was not affected (Fig. 4A). This result is consistent with the notion that polyubiquitination is involved in TRAF6-induced IKK activation but not in MEKK1-induced IKK activation (*9*). More importantly, both ubiquitin mutants suppressed Tax-induced IKK activation (Fig. 4A). This result indicates that polyubiquitination is involved in Tax-induced IKK complex activation.

As Tax binding to NEMO is essential for Tax-induced IKK activation (*22*, *23*), we next examined whether the ubiquitin mutants inhibited the interaction between Tax and the IKK complex. The Jurkat cytosolic extract was incubated with recombinant Tax with and without either wild-type ubiquitin or the mutants. An aliquot of the reaction mixture was immunoblotted directly to examine IKK activation, and the remaining mixture was used for Ni-NTA pull-down assays to analyse the interaction between Tax and the IKK complex. Although K0-Ub and Me-Ub inhibited Tax-induced IKK activation as expected (Fig. 4B, left), the addition of these mutants had no effect on the association between Tax and the IKK complex (Fig. 4B, right). These results suggest that the interaction between Tax and the IKK complex is insufficient to induce IKK activation, and there may be additional steps, in which polyubiquitination is involved, for Tax-induced activation of the IKK complex.

As the addition of K0-Ub does not inhibit the generation of linear polyubiquitin chains (*36*), K0-Ub-mediated inhibition of Tax-induced IKK activation suggests that linear polyubiquitination is

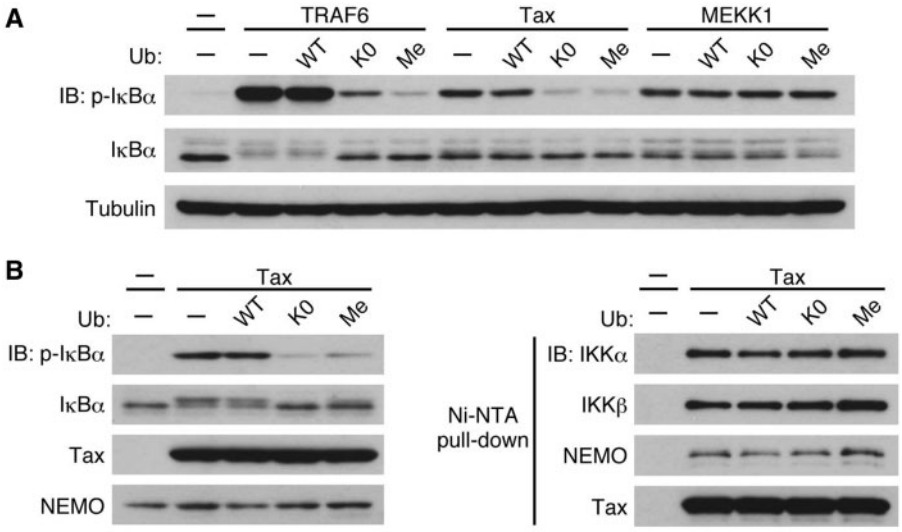

Fig. 4 Polyubiquitination is involved in Tax-induced IKK activation. (A) The Jurkat cytosolic extract (10 mg/ml) was mixed with wild-type (WT, 100 μM), lysine-free (K0, 100 μM), or methylated (Me, 100 μM) ubiquitin and then incubated with recombinant TRAF6 (0.5 μM), Tax (0.5 μM) or MEKK1 (0.5 μM) and ATP (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody. (B) The Jurkat cytosolic extract (10 mg/ml) was mixed with either WT ubiquitin or the ubiquitin mutants (100 μM), as well as recombinant Tax (0.5 μM) and ATP (2 mM) at 30°C for 1 h. After incubation, aliquots of the reaction mixtures were immunoblotted directly with an anti-p-IκBα antibody to examine IKK activation, and the remaining mixture was used for a Ni-NTA pull down to analyse the interaction between Tax and the IKK complex by immunoblot with anti-IKKα, anti-IKKβ, anti-NEMO and anti-Tax antibodies.

dispensable or insufficient for IKK activation by Tax. These results led us to test whether Lys63-linked polyubiquitination is involved. We first examined whether Tax induces polyubiquitination in the cell-free system. Immunoprecipitation and subsequent immunoblot with an anti-ubiquitin antibody revealed that Tax induces polyubiquitination of Tax and NEMO in the cell-free system (Fig. 5A). The cell-free assay was then performed with the ubiquitin mutant, either K48R or K63R, in which the Lys-48 or Lys-63 of ubiquitin was mutated to Arg, respectively. K48R did not have an effect, but K63R completely inhibited Tax-induced IKK activation (Fig. 5B), which indicates that Lys-63-linked polyubiquitination is required for Tax-induced IKK activation. In the IL-1R and TLR pathways, TRAF6 acts as an E3 ubiquitin ligase to generate K63-linked polyubiquitin chains upon stimulation (*8*, *9*). Therefore, cytosolic extracts were prepared from wild-type MEF cells and *Traf6*$^{-/-}$ MEF cells and then tested for Tax-induced IKK activation. Similar levels of IκBα phosphorylation were observed in the WT and *Traf6*$^{-/-}$ extracts (Fig. 5C), which indicates that TRAF6 is dispensable for Tax-induced IKK activation.

In this study, using a cell-free assay system, we demonstrated that activation of the IKK complex by HTLV-1 Tax requires cytosolic factor(s) that are involved in Tax-induced polyubiquitination. As polyubiquitination is catalysed by three enzymes, E1, E2 and E3 (*37*), it is important to identify these enzymes to further elucidate the molecular mechanisms of Tax-induced IKK activation. Although our results strongly suggest that K63-linked polyubiquitin chains are involved in the Tax-induced IKK activation, TRAF6 does not act as an E3 and the involvement of Ubc13 as an E2 is still controversial (*15*, *16*). It is well known that polyubiquitin chains with different lysine linkages have distinct biological roles (*38*, *39*). Lys48-linked polyubiquitin chains are a signal for proteasomal degradation. On the other hand, Lys63-linked and linear polyubiquitin chains play an important role in the cytokine signal transduction pathway that leads to NF-κB activation. Further studies are required to determine which types of polyubiquitin chain are involved in Tax-induced IKK complex activation. We also found that polyubiquitination was not required for the interaction between Tax and the IKK complex. This result raises the possibility that polyubiquitination might be required either to recruit IKK kinase (IKKK) or to induce *trans*-autophosphorylation of the IKK complex, which induces IKK activation. It has been reported that ubiquitination of Tax is required for the interaction of Tax with NEMO (*16*). Although the reason for the discrepancy

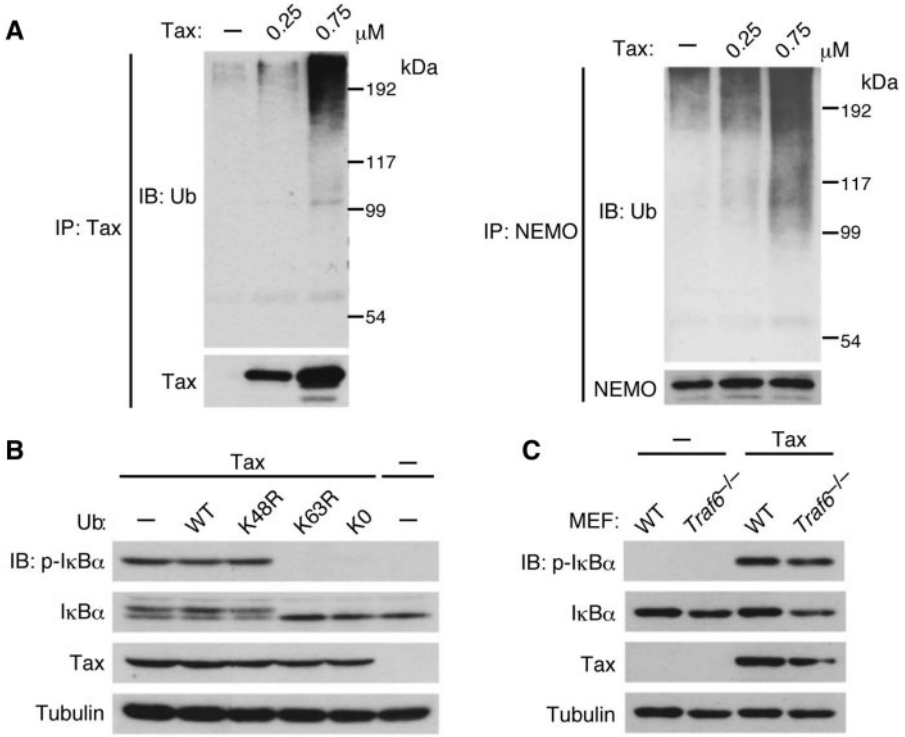

Fig. 5 Potential involvement of Lys-63-linked polyubiquitination in Tax-induced IKK activation. (A) Recombinant Tax induces polyubiquitination of Tax and NEMO in the cell-free system. The Jurkat cytosolic extract (10 mg/ml) was incubated with recombinant Tax with ATP (2 mM) at 30°C for 1 h. After incubation, the reaction mixture was first boiled to remove the non-covalently attached proteins and then immunoprecipitated with either an anti-Tax (left) or anti-NEMO (right) antibody. Ubiquitination of Tax and NEMO was detected by immunoblot with an anti-ubiquitin antibody. (B) The Lys63-linked polyubiquitin chain is involved in Tax-induced IKK activation. The Jurkat cytosolic extract (10 mg/ml) was incubated with recombinant Tax (0.5 μM) and either the WT or ubiquitin mutants (100 μM) with ATP (2 mM) at 30°C for 1 h. IκBα phosphorylation was detected by immunoblot with an anti-p-IκBα antibody. (C) TRAF6 is not involved in Tax-induced IKK activation. Recombinant Tax (0.5 μM) was incubated with the cytosolic extract from either WT or *Traf6*$^{-/-}$ MEFs (10 mg/ml) with (2 mM) at 30°C for 1 h. Phosphorylation of IκBα was detected by immunoblot with an anti-p-IκBα antibody.

is not clear, identification of the polyubiquitinated proteins and IKK-interacting proteins using our cell-free system may provide clues to understanding the molecular mechanisms of Tax-induced IKK activation.

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Conflict of interest

None declared.

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