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HTLV-1 Tax-induced NFκB activation is independent of Lys-63-linked-type polyubiquitination

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) Tax-induced activation of nuclear factor-κB (NFκB) is thought to play a critical role in T-cell transformation and onset of adult T-cell leukemia. However, the molecular mechanism of the Tax-induced NFκB activation remains unknown. One of the mitogen-activated protein kinase kinase kinses (MAP3Ks) members, TAK1, plays a critical role in cytokine-induced activation of NFκB, which involves lysine 63-linked (K63) polyubiquitination of NEMO, a noncatalytic subunit of the IκB kinase complex. Here we show that Tax induces K63 polyubiquitination of NEMO. However, TAK1 is dispensable for Tax-induced NFκB activation, and deubiquitination of the K63 polyubiquitin chain failed to block Tax-induced NFκB activation. In addition, silencing of other MAP3Ks, including MEKK1, MEKK3, NIK, and TPL-2, did not affect Tax-induced NFκB activation. These results strongly suggest that unlike cytokine signaling, Tax-induced NFκB activation does not involve K63 polyubiquitination-mediated MAP3K activation.
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Keywords: HTLV-1; Tax; NFκB; TAK1; MAP3K; NEMO; Ubiquitination

Human T-cell leukemia virus type 1 (HTLV-1) infects and immortalizes $CD4^+$ T cells in vitro and is etiologically associated with adult T-cell leukemia (ATL) [1]. The nonstructural Tax protein of HTLV-1 is crucial for regulation of viral replication and for initiating the malignant transformation that leads to the development of ATL. Tax activates intracellular signal transduction pathways that normally play a crucial role in cellular responses to various extracellular stimuli, resulting in the activation of several transcription factors, including nuclear factor-κB (NFκB), cAMP-responsible element binding protein (CREB), and serum response factor (SRF) [1,2]. Several lines of evidence strongly suggest that Tax-induced activation of NFκB plays a critical role in the onset of ATL. Recombinant HTLV-1 virus carrying the Tax M22 mutant, which activates CREB but not NFκB, is unable to immortalize T cells, whereas the M47 mutant, which activates NFκB but not CREB, induces T-cell immortalization [3]. NFκB activation induces expression of genes required for T-cell growth, such as IL-2Rα chain [4]. NFκB essential modifier (NEMO), a subunit of the protein kinase complex essential for NFκB activation, is inactivated in flat, spontaneously reverted cells derived from Tax-transformed rat fibroblasts [5]. Tax-induced activation of NFκB causes inactivation of p53 protein, which is involved in cell-cycle checkpoint regulation and is inactivated in many cancers [6]. Therefore,

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identification of the molecular mechanism(s) by which Tax activates NFκB may lead to development of drugs to prevent onset of ATL in HTLV-1-infected individuals [7].

NFκB is sequestered in the cytoplasm due to association with its inhibitory proteins, IκBα, IκBβ, and IκBε. When cells are stimulated with cytokines, pathogens, mitogens, or stress inducers, IκB proteins are phosphorylated at specific serine residues. These phosphorylated IκB proteins are then ubiquitinated and targeted for proteasome-dependent degradation. The free NFκB translocates to the nucleus where it transactivates various target genes involved in immune responses and cellular proliferation and differentiation. IκB is phosphorylated by activated IκB kinase (IKK) complex, which includes two catalytic subunits, IKKα and IKKβ and NEMO. Several studies have shown that IKK activation induced by signaling from the interleukin-1 receptor (IL-1R) family, Toll-like receptor (TLR) family, tumor necrosis factor receptor (TNFR), and T-cell receptor (TCR) requires lysine 63 (K63)-linked polyubiquitination of signaling molecules, such as TNFR-associated factor (TRAF) family proteins, receptor interacting protein kinase 1 (RIP1), and NEMO [8–11]. TRAF6 acts as an E3 ubiquitin-ligase to catalyze the K63-linked polyubiquitination of NEMO and TRAF6 itself through its own RING finger domain in TLR/IL-1R signaling, whereas TRAF2 catalyzes polyubiquitination of RIP1 and NEMO [8,9]. Polyubiquitinated TRAF6 and RIP-1 then recruit the transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) complex through the interaction of the polyubiquitin chains and the zinc-finger domain of TAK1-binding protein 2 (TAB2) or TAB3 [9]. IKKβ in the complex is then phosphorylated and activated by TAK1, leading to phosphorylation of IκB.

In this paper, we examine whether Tax mimics the actions of any one of the molecules in the ubiquitination-dependent NFκB activation pathway to activate the IKK complex, because Tax activates NFκB in a NEMO-dependent manner.

Materials and methods

Cell culture and antibodies (Abs). Jurkat, JPX-9, Hut102, Hayai, MT-2, and MT-4 cells were grown in RPMI1640 medium containing heat-inactivated 10% FBS. HEK293T cells and MEFs from wild-type and TAK1-deficient mice were grown in DMEM containing heat-inactivated 10% FBS. Anti-TAK1, anti-NEMO, anti-TRAF6, anti-Myc, and anti-HA Abs were purchased from Santa Cruz Biotechnology. Anti-FLAG Ab was from Sigma–Aldrich. Anti-phospho-IKKβ and anti-IKKβ Abs were obtained from Cell Signaling Technology. Anti-Ubc13 and anti-tubulin Abs were purchased from Zymed Laboratories and Calbiochem, respectively. Anti-Tab1 Ab was kindly provided by Dr. Matsumoto (Nagoya University, Japan).

Detection of polyubiquitination of NEMO. HEK293T cells were co-transfected with the indicated combinations of pcDNA3-6xMyc-Ub (or HA-Ub) mutant (K63R) and the indicated expression vectors. After 48 h incubation, cells were lysed in TNE buffer containing 1% SDS and boiled for 5 min. The lysates were diluted with a 10-fold volume of TNE buffer and subjected to immunoprecipitation with anti-NEMO antibody and protein G-Sepharose (Amersham Pharmacia). The polyubiquitinated NEMO was detected by immunoblotting with anti-Myc antibody.

Immunoblotting. Cell lysates were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were incubated with various primary Abs and then incubated with anti-mouse or anti-rabbit IgG Ab linked to HRP (Amersham Pharmacia). Immunocomplexes were visualized with an ECL Plus Detection System (Amersham Pharmacia).

NFκB luciferase reporter assay. HEK293 T cells were transfected with 5 ng of 3xκB-luc, 50 ng of β-actin-β-gal, and the indicated amounts of various expression plasmids by a calcium phosphate method. After 24 h incubation, luciferase activity was measured with a PicaGene Luciferase Assay System (Toyo Ink), and β-galactosidase activity was used to standardize the transfection efficiency.

EMSA. Binding reaction mixture containing nuclear extract and ^{32}P-labeled probe were incubated for 20 min at room temperature. DNA-protein complexes were separated by polyacrylamide gel electrophoresis and visualized by autoradiography.

Retrovirus-mediated gene transfer. Plat-E cells were transfected with a pMX retrovirus vector encoding *tax* cDNA with Lipofectamine 2000 (Invitrogen). Virus stocks were prepared by collecting the culture media 48 h after transfection. MEFs were incubated in virus stock medium containing 10 μg/ml polybrene for 4 h and further incubated for 48 h.

RNAi and RT-PCR. HEK293 T cells were transfected with Stealth RNAi Negative Control Duplexes (Invitrogen) and Stealth siRNA (Invitrogen) with Lipofectamine 2000. For RT-PCR, total cDNA was synthesized from total RNA extracted from the cells. Semiquantitative PCR with primers specific for MEKK1, MEKK3, NIK, TPL-2, NEMO, and β-actin was conducted.

Results and discussion

Tax activates NFκB without activating TAK1

Because TAK1 is involved in NFκB activation induced by various stimuli through phosphorylation of IKKβ, we first examined the activity of TAK1 in HTLV-1-transformed cell lines. Activation of TAK1 coincides with phosphorylation of TAB1, which results in the mobility shift of the protein on SDS–polyacrylamide gels [12]. Phosphorylation of TAB1 was not observed in cell lysates of unstimulated Jurkat cells, whereas phosphorylated TAB1 was detected in lysates prepared from HTLV1-transformed cell lines that express Tax (Hut102, Hayai, MT-2, and MT-4) (Fig. 1A). These results strongly suggest that TAK1 is constitutively activated in many HTLV-1-transformed cell lines, which led us to hypothesize that Tax activates NFκB through TAK1 activation. To examine whether Tax activates TAK1, JPX-9 cells, a derivative of Jurkat cells in which the *tax* gene is under the control of the metallothionein promoter, were treated with $CdCl_2$ to induce Tax expression. When JPX-9 cells were treated with $CdCl_2$, Tax expression was induced, and nuclear translocation of NFκB was detected by EMSA (Fig. 1B). However, the phosphorylated form of TAB1 was not observed, indicating that Tax activates NFκB without TAK1 activation (Fig. 1B).

Next, we investigated whether TAK1 is required for Tax-induced NFκB activation using siRNAs that silence TAK1 expression. HEK293T cells were first transfected with TAK1-specific siRNA. Cells were then transfected with NFκB reporter plasmid and various amounts of Tax expression vector (Fig. 1C). TAK1-specific siRNA signifi-

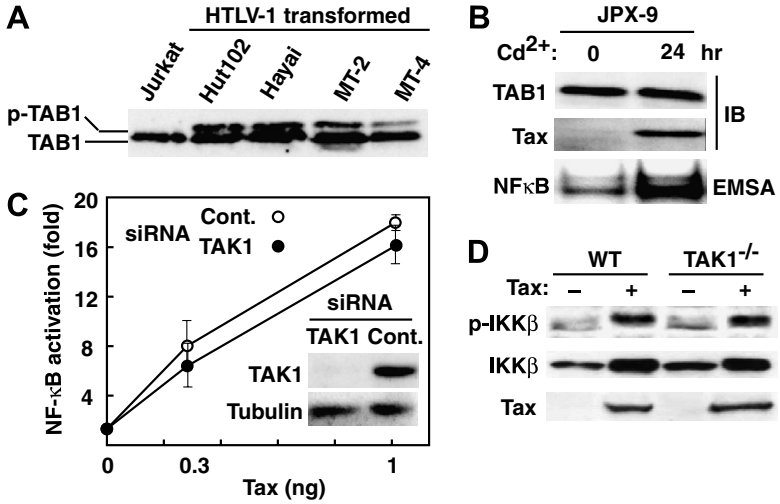

Fig. 1. TAK1 is constitutively activated in HTLV-1-transformed cell lines independently of Tax. (A) Lysates of the indicated cells were analyzed by immunoblotting with anti-TAB1 Ab. (B) JPX-9 cells were incubated with or without $CdCl_2$ (20 μM) for 24 h. Cell lysates were analyzed by immunoblotting (IB) with anti-TAB1 and anti-Tax (Lt-4) Abs. Nuclear extracts were subjected to EMSA with an NFκB-binding probe. (C) HEK293T cells were transfected with *TAK1*-specific siRNA or control siRNA. After 48 h incubation, cell lysates were analyzed by immunoblotting with anti-TAK1 and anti-tubulin Abs. The cells were transfected with a 3xκB-luc and the indicated amounts of pCG-Tax at 48 h after siRNA-transfection. After further 24 h incubation, the cells were harvested for luciferase assay. The results are shown as fold-induction over luciferase activity of the untransfected cells. (D) Wild-type- and *TAK1*-deficient MEFs were infected with retrovirus expressing Tax as described in Materials and methods. Cell lysates were analyzed by immunoblotting with anti-phosphorylated IKKβ (p-IKKβ), anti-IKKβ, and anti-Tax Abs.

cantly reduced expression of TAK1 protein (Fig. 1C, inset). However, no significant difference was observed in NFκB-driven transcriptional activity between *TAK1* siRNA- and control siRNA-treated cells. These results strongly suggest that TAK1 is dispensable for NFκB-dependent transcription induced by Tax.

To confirm further that TAK1 is not required for Tax-induced NFκB activation, Tax-induced phosphorylation of IKKβ, which reflects IKK activation, was analyzed in TAK1-deficient ($TAK1^{-/-}$) and wild-type (WT) MEFs. When the *tax* gene was retrovirally transduced into $TAK1^{-/-}$- and WT-MEF, IKKβ was phosphorylated irrespective of TAK1 expression (Fig. 1D). Taken together, these data indicate that TAK1 is not necessary for Tax-induced NFκB activation.

The observation that TAK1 is constitutively activated in many HTLV-1-transformed cell lines is interesting. This activation appears to occur during transformation of T cells by HTLV-1 infection because TAK1 is not activated in resting T cells, and Tax cannot activate TAK1 directly. Therefore, constitutive activation of NFκB in Tax-deficient ATL cells [2], which is essential for survival of ATL cells, may be due to constitutive activation of TAK1. Furthermore, c-Jun N-terminal kinase (JNK), which can be activated by TAK1, is constitutively activated in both Tax-expressing HTLV-1-transformed cells and Tax-deficient ATL cells [13]. Therefore, TAK1 could contribute to Tax-independent activation of NFκB and JNK in ATL cells. Constitutive activation of TAK1 in ATL cells could be involved in survival and malignant characteristics of ATL cells in vivo, which is supported by a previous report of the oncogenic properties of TAB3 [14]. Further studies are needed to clarify the roles and mechanisms of TAK1-mediated signaling pathways in ATL cells.

Tax induces K63-linked polyubiquitination of NEMO

NEMO is required for IKKβ activation induced by cytokines, pathogens, and other ligands that lead to NFκB activation. Stimulation by these ligands induces K63 polyubiquitination of NEMO, which is catalyzed by TRAF6 [11]. Furthermore, stimulation of TCRs results in the formation of a protein complex that is composed of CARMA1, Bcl-10, and MALT1 followed by conjugation of the K63 polyubiquitin chain to NEMO at K399, leading to activation of IKK complex [10]. Therefore, K63 polyubiquitination of NEMO could be a crucial step in IKK activation.

Because NEMO is required for IKKβ activation induced by Tax, we examined whether Tax induces K63 polyubiquitination of NEMO. NEMO was co-expressed with a Myc-tagged ubiquitin mutant containing only one lysine at position 63 (Myc-K63Ub) in the absence or presence of Tax or TRAF6 in HEK293T cells. NEMO was then immunoprecipitated and immunoblotted with anti-Myc antibody to detect polyubiquitinated NEMO (Fig. 2A). In the present study, TRAF6 efficiently induced K63 polyubiquitination of NEMO as described previously [11]. Interestingly, K63 polyubiquitination of NEMO was also induced by Tax expression (Fig. 2A).

We next performed two distinct experiments to address whether K63 polyubiquitination of NEMO is required for Tax-induced NFκB activation. We first examined the requirement for Ubc13, a component of E2 essential for

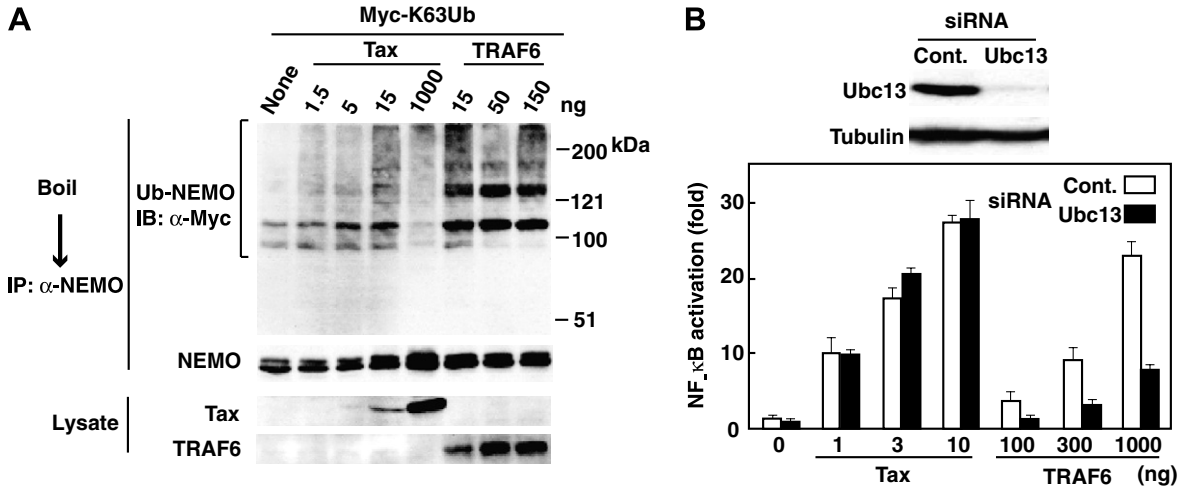

Fig. 2. Tax induces lysine 63-linked polyubiquitination of NEMO. (A) pcDNA3-6xMyc-K63Ub (500 ng) and pRK5-FLAG-NEMO (500 ng) were co-transfected into HEK293 T cells with the indicated amounts of pCG-Tax or pME-FLAG-TRAF6. Ubiquitinated NEMO was detected as described in Materials and methods. The two intense bands above 100 and 120 kDa indicate tri- and tetra-ubiquitinated NEMO, respectively. Ten percent of total lysates were analyzed by immunoblotting with anti-Tax or anti-FLAG (for TRAF6) Ab. (B) HEK293 T cells were transfected with Ubc13-specific siRNA or control siRNA. After 48 h incubation, cell lysates were analyzed by immunoblotting with anti-Ubc13 and anti-tubulin Abs. The cells were transfected with 3xκB-luc and the indicated amounts of pCG-Tax or pME-FLAG-TRAF6 at 48 h after siRNA-transfection. After further 24 h incubation, the cells were harvested for the luciferase assay. The results are displayed as a fold induction over luciferase activity of the untransfected cells.

K63 polyubiquitination [8], in Tax-induced NFκB activation. HEK293T cells were transfected with Ubc13 siRNA. Cells were then co-transfected with the NFκB reporter construct and Tax or TRAF6 expression vector. Introduction of Ubc13 siRNA severely reduced Ubc13 expression (Fig. 2B, upper panel). Although TRAF6-induced NFκB activation was significantly impaired when Ubc13 expression was suppressed as described previously [10], Tax-induced activation was not affected significantly (Fig. 2B, lower panel).

We then examined the effect of deubiquitination on Tax-induced NFκB activation. The tumor suppressor cylindromatosis (CYLD) inhibits NFκB activation by deubiquitinating K63 polyubiquitin chains on TRAF2, TRAF6, and NEMO [15–17]. We co-transfected the NFκB luciferase reporter plasmid with either the Tax, TRAF2, or TRAF6 expression vector together with different amounts of CYLD expression vector. Expression of CYLD suppressed the NFκB activation by TRAF2 or TRAF6 in a dose-dependent manner as described previously [15–17], whereas it had no effect on Tax-induced NFκB activation (Fig. 3A). Similar experiments with the CREB luciferase reporter instead of the NFκB reporter revealed that K63 ubiquitination is not required for Tax-induced CREB activation (data not shown). To confirm that Tax- or TRAF6-induced polyubiquitination of NEMO is blocked by CYLD expression, NEMO and an HA-tagged ubiquitin mutant (HA-K63Ub) were co-expressed with Tax or TRAF6 in the presence or absence of CYLD. Expression of CYLD significantly reduced K63 polyubiquitination of NEMO induced by either Tax or TRAF6 (Fig. 3B). These results indicate that Tax can induce K63 polyubiquitination of NEMO but that such polyubiquitination is not necessary for Tax-induced NFκB activation. Although it has been reported that Tax induces polyubiquitination of NEMO [18], the type of the polyubiquitin chain has not been identified, and the role of this chain in NFκB activation has not been addressed. In this paper, we show for the first time that Tax, like the TRAF proteins, induces K63 polyubiquitination of NEMO, and our results strongly suggest that Tax-induced K63 ubiquitination is not essential for Tax-induced NFκB activation. However, we cannot exclude the possibility that K63 polyubiquitination of a small amount of NEMO that is below the limit of detection of our immunoblotting method is sufficient for NFκB activation. It is not known whether Tax causes K63 ubiquitination of proteins other than NEMO. Polyubiquitin chains on such proteins could be stable in the presence of CYLD and could be involved in Tax-induced NFκB activation. Formation of other types of polyubiquitin chain might be necessary for Tax-induced NFκB activation. It has been reported that Tax-mediated NFκB activation is blocked by expression of A20 [19], a deubiquitinase that degrades K63- and K48- linked polyubiquitin chains [20]. Therefore, the K48-type polyubiquitin chain, which usually acts as a marker recognized by proteasomes, could be formed on some proteins, leading to activation of NFκB. On the basis of data from the experiments with various Tax mutants in which individual lysine residues were systematically replaced with arginine to block ubiquitination [21], it has been suggested that ubiquitination of Tax itself is involved in NFκB activation. Because introduction of such mutations into Tax could cause conformational changes in the protein and alter structures necessary for NFκB activation in addition to blockade of ubiquitination, further experiments are needed.

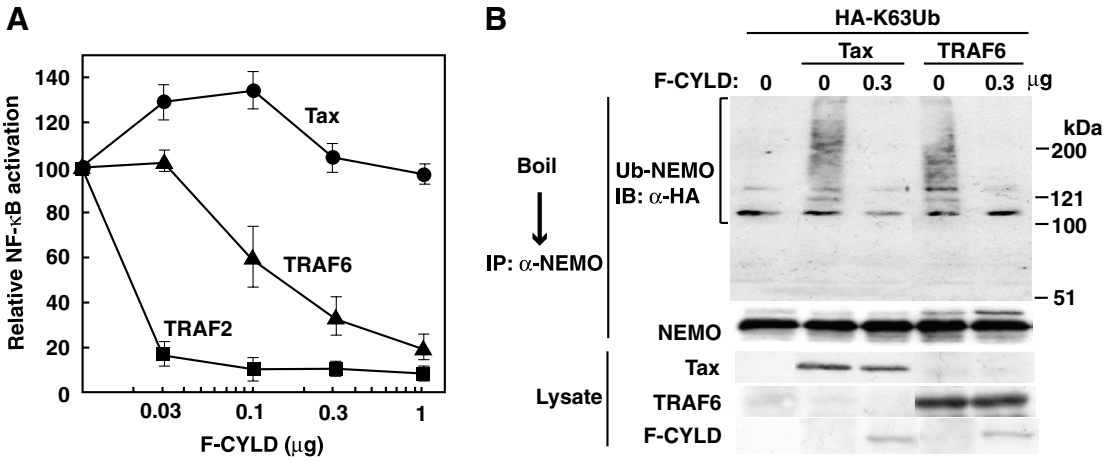

Fig. 3. K63-linked polyubiquitination of NEMO is not necessary for Tax-induced NFκB activation. (A) 3xκB-luc and the indicated amounts of expression vector encoding FLAG-tagged CYLD were co-transfected into HEK293 T cells with pCG-Tax (10 ng), pME-FLAG-TRAF2 (300 ng), or TRAF6 (300 ng). After 24 h incubation, the cells were harvested for luciferase assay. The results are reported as the percentage of the luciferase activity of CYLD-untransfected cells. (B) Expression vector encoding HA-K63Ub (0.5 μg), FLAG-NEMO (0.5 μg), or FLAG-CYLD (0.3 μg) was co-transfected into HEK293 T cells with pCG-Tax (10 ng) or pME-FLAG-TRAF6 (300 ng). Ubiquitinated NEMO was detected as described in Materials and methods. Ten percent of each lysate was analyzed by immunoblotting with anti-Tax or anti-FLAG (for TRAF6 and CYLD) Ab.

Tax activates NFκB independent of MEKK1, MEKK3, NIK, and TPL-2

Because Tax induces phosphorylation of IKKβ at two critical serine residues in the activation loop [22], it is possible that Tax-induced NFκB activation is mediated by MAP3K other than TAK1, the best characterized IKK-kinase (IKKK) [23]. Several lines of evidence suggest that MEKK1, MEKK3, NIK, and TPL-2 could belong to the IKKK family [23]. Previous studies showed that MEKK1, NIK, and TPL-2 are involved in Tax-mediated NFκB activation [24–26]. However, there is no definitive evidence to indicate that Tax activates NFκB via these kinases, because the authors' conclusions were based on results from over-expression studies. Therefore, we examined whether silencing of MEKK1, MEKK3, NIK, or TPL-2 affects Tax-induced NFκB activation. HEK293T cells were transfected with siRNA targeted to one of these four MAP3Ks or NEMO. The cells were then co-transfected with NFκB luciferase reporter plasmid and various amounts of Tax expression vector. RT-PCR of total RNAs from siRNA-treated cells revealed that levels of the *MEKK1*, *MEKK3*, *NIK*, *TPL-2,* and *NEMO* mRNAs were significantly lower than those in control siRNA-treated cells (Fig. 4A). Knockdown of NEMO, which is essential for Tax-mediated NFκB activation, caused severe inhibition of NFκB-driven transcription activated by Tax (Fig. 4B). However, silencing of MEKK1, MEKK3, NIK, or TPL-2 had no effect on Tax-induced NFκB activation (Fig. 4B). These results strongly suggest that Tax-induced NFκB activation is not mediated by MEKK1, MEKK3, NIK, or TPL-2. These studies raise the possibility that Tax could induce autophosphorylation of IKKβ in either a *cis*- or *trans*-manner by changing the conformation of the IKK complex or by inactivating a putative IKK inhibitor to activate the IKK complex.

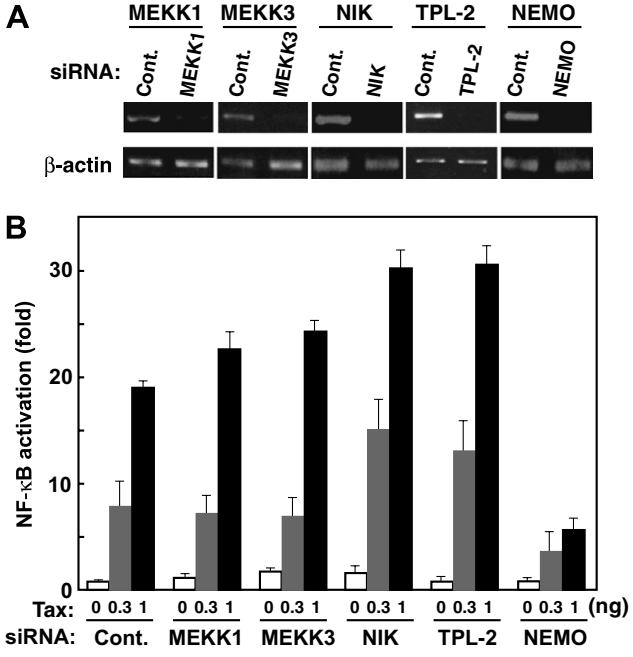

Fig. 4. Tax induces NFκB activation independent of MEKK1, MEKK3, NIK, or TPL-2. (A) HEK293T cells were transfected with siRNA targeted to the indicated mRNAs or control siRNA. After 48 h incubation, total RNA was subjected to semi-quantitative RT-PCR. (B) At 48 h after transfection with the siRNAs described in (A), the cells were transfected with 3xκB-luc and the indicated amounts of pCG-Tax. After 24 h incubation, the cells were harvested for luciferase assay. The results are shown as fold induction over luciferase activity of the untransfected cells.

In conclusion, our present results strongly suggest that unlike the cytokine signaling mediated by members of the TRAF family, Tax-induced NFκB activation may be mediated by novel molecular mechanisms that do not involve K63 polyubiquitination. Further studies are needed to clarify the mechanism(s) of Tax-induced NFκB activation.

Acknowledgments

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