Polyubiquitination of Transforming Growth Factor β (TGFβ)-associated Kinase 1 Mediates Nuclear Factor-κB Activation in Response to Different Inflammatory Stimuli*

Anahita Hamidi†1, Verena von Bulow†1, Rosita Hamidi§, Nicolas Winssinger§, Sofia Barluenga§, Carl-Henrik Heldin†, and Marene Landström¶1,2

From the †Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden, the §Organic and Bioorganic Laboratory, University of Strasbourg, CNRS (UMR7006), Strasbourg, France, and the ¶Medical Biosciences, Umeå University, 90185 Umeå, Sweden

Background: Activation of TAK1 is a key event in inflammation.

Results: Different cytokines cause TRAF6-dependent polyubiquitination at Lys-34 of TAK1 and activation of the TAK1-NF-κB pathway.

Conclusion: Interestingly, this event is common for several different pathways.

Significance: The knowledge of how activation of the TAK1-NF-κB pathway occurs might aid to create novel drugs to combat inflammation and cancer.

The transcription factor nuclear factor κB (NF-κB) plays a central role in regulating inflammation in response to several external signals. The TGFβ-associated kinase 1 (TAK1) is an upstream regulator of NF-κB signaling. In TGFβ-stimulated cells, TAK1 undergoes Lys-63-linked polyubiquitination at Lys-34 by TNF receptor-associated factor 6 and is thereby activated. The aim of this study was to investigate whether TAK1 polyubiquitination at Lys-34 is also essential for NF-κB activation via TNF receptor, IL-1 receptor and toll-like receptor 4. We observed that TAK1 polyubiquitination occurred at Lys-34 and required the E3 ubiquitin ligase TNF receptor-associated factor 6 after stimulation of cells with IL-1β. Polyubiquitination of TAK1 also occurred at Lys-34 in cells stimulated by TNF-α and LPS, which activates TLR4, as well as in HepG2 and prostate cancer cells stimulated with TGFβ, which in all cases resulted in NF-κB activation. Expression of a K34R-mutant TAK1 led to a reduced NF-κB activation, IL-6 promoter activity, and proinflammatory cytokine secretion by TNF-α-stimulated PC-3U cells. Similar results were obtained in the mouse macrophage cell line RAW264.7 after LPS treatment. In conclusion, polyubiquitination of TAK1 is correlated with activation of TAK1 and is essential for activation of NF-κB signaling downstream of several receptors.

Members of the family of nuclear factor-κB (NF-κB) transcription factors regulate expression of a large number of genes involved in immune and inflammatory responses, as well as in cell survival, proliferation, and differentiation (1). Toll-like receptors (TLRs), TNF receptor (TNF-R) and IL-1 receptor (IL-1R) play important roles in regulating the activity of NF-κB in innate immunity. Inappropriate activation of NF-κB signaling has been implicated in the pathogenesis of chronic inflammation, autoimmunity, and various cancers. The active NF-κB complex is composed of homo- and heterodimers of the NF-κB/Rel family. In the classical NF-κB pathway, the NF-κB dimers p50/p65 are retained in an inactive form in the cytoplasm by the NF-κB inhibitor IκBα. Upon stimulation of cells with, for example, TNF-α, IL-1β, or LPS, the IκB kinase (IKK) complex, which consists of two catalytic subunits, IKKa and IKKβ, and a regulatory subunit, IκKγ/NEMO, is activated. Activated IKK phosphorylates IκBα and targets it for Lys-48 polyubiquitination and rapid degradation. Free p50/p65 can thereafter translocate into the nucleus and coordinate the transcription of its target genes (1).

The TGFβ-associated kinase 1 (TAK1) has emerged as a key regulator of signal transduction cascades leading to activation of NF-κB. TAK1 is a member of the mitogen-activated protein kinase kinase kinase family and was originally identified to play an essential role in the TGFβ pathway (2). Later, TAK1 was also identified in the TNF-R-, IL-1R- and TLR-mediated signaling pathways as a potent activator of IKK signaling (3). Recently, we and others described the molecular mechanism of TAK1 activation in the TGFβ signaling pathway (4, 5). The E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) interacts with a consensus motif present in TGFβ type I receptor (TβRI). TGFβ-induced oligomerization of TβRI induces autoubiquitination of TRAF6 and Lys-63-linked polyubiquitination of TAK1 at Lys-34, which correlates with TAK1 activation and activation of the downstream p38 MAP kinase (4).
TAK1 Polyubiquitination Is Critical for NF-κB Activation

Over the past years it has become clear that an important key mechanism of regulation of the NF-κB signaling pathway involves posttranslational modifications of key proteins via ubiquitin chains (6). Besides Lys-48-linked ubiquitin chains marking proteins for proteasomal degradation, the roles of Lys-63-linked (7) and linear (8) polyubiquitination in NF-κB activation have recently been documented. Site-specific Lys-63-linked auto-ubiquitination of TRAF6 is a critical upstream mediator of IKK activation in IL-1R signaling (9). Auto-ubiquitination of TRAF6 occurs also in a TLR4-dependent manner (10). In the TNF-α signaling pathway, phosphorylation of TRAF2 by protein kinase C determines Lys-63-linked polyubiquitination of TRAF2, thereby inducing association with TAK1 binding proteins (TAB) 2 and 3 and bridging of the TAK1 complex to IKKα/β (11). TAK1 has also been found to be autoactivated in complex with TAB1, presumably because of the close structural similarity between TAB1 and protein phosphatase 2Ca (PP2Ca) (12–14).

We investigated in this study whether polyubiquitination of TAK1 is a common molecular mechanism for its activation in three different inflammatory pathways, i.e. signaling via TNFR, IL-1R, and TLR4. We report here that polyubiquitination of TAK1 at Lys-34 is required for appropriate NF-κB signaling in all three pathways. TAK1 mutated at Lys-34 impaired NF-κB activation by preventing nuclear translocation of p65, leading to down-regulation of IL-6 gene expression and reduced cytokine secretion in TNF-α- and LPS-stimulated cells.

EXPERIMENTAL PROCEDURES

Cell lines, Reagents, and Antibodies—HEK293, human heptoma HepG2 cells and mouse macrophage RAW264.7 cells were obtained from the ATCC. HEK293–IL-1R cells were a gift from J. Ninomiya-Tsujii (Department of Environmental and Toxicology, North Carolina State University). HEK293–TLR4 cells (clone no. BD11) were a gift from A. B. Schromm (Research Center Borstel, Germany) and were established by transfection of HEK293 cells with expression plasmids coding for full-length human TLR4 (the expression plasmid for TLR4 was a kind gift of Douglas Golenbock, University of Massachusetts Medical School, Worcester, MA). Transfected cells were selected in the presence of geneticin (G418). Stable clones were generated by limited dilution technique and maintained at 37 °C under an atmosphere of 5% CO₂ in DMEM containing 10% FCS, Linaris, Bettingen am Main, Germany), 0.5 units/ml penicillin, 0.5 μg/ml streptomycin (Biochrom AG, Berlin, Germany), and 0.4 mg/ml G418.

The HEK293, HepG2, and RAW264.7 cell lines were cultured in DMEM (Sigma-Aldrich) containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293–TLR4 cells were cultured in DMEM containing 10% FCS, 2 mM L-glutamine, 0.4 μg/ml G418, 100 units/ml penicillin, and 100 μg/ml streptomycin. The human prostate carcinoma cell line PC-3U, originating from PC-3 cells (15), was grown in RPMI 1640 medium containing 10% FCS and 2 mM L-glutamine. Transient transfections of HEK293 cells were performed using the calcium phosphate method. PC-3U and RAW264.7 cells were transiently transfected using FuGENE 6 (Roche) as described previously (16). Cells were starved at least 16 h before stimulation in 3% FCS (WT or transfected HEK293 cells) or 1% FCS (PC-3U cells). Cells were stimulated by adding 10 ng/ml TNF-α (Immunokontakt), 10 ng/ml IL-1β (Sigma-Aldrich), 10 ng/ml TGFβ1 (R&D Systems), or 500 ng/ml LPS (Sigma-Aldrich) to the starvation media. For immunoprecipitation experiments, mouse monoclonal anti-FLAG M2 (Sigma) and anti-HA (12CA5) (Roche) were used. Polyclonal rabbit IKKα/β (H470), polyclonal rabbit HA (Y11), polyclonal rabbit TAK1, monoclonal mouse ubiquitin (P4D1), and c-Myc were purchased from Santa Cruz Biotechnology, Inc. Polyclonal rabbit lamin A/C, monoclonal rabbit anti-phospho-IKKα/β (Ser-176/180, 16A6), polyclonal rabbit anti-1κBα, and monoclonal mouse anti-phospho-1κBα (Ser-32/36, 5A5) were from Cell Signaling Technology, Inc.

Monoclonal mouse anti-β-actin and monoclonal rabbit anti-p65 were obtained from Abcam. Polyclonal rabbit β-tubulin was obtained from Sigma. The phospho-specific rabbit antisera against phosphorylated and activated TAK1 (phospho-Thr-187/Ser-192) was generated in our laboratory and described previously (4). Alexa Fluor 488 was purchased from Invitrogen.

Inhibitors—The TAK1 inhibitor chloro-radicicol A, compound 31, was prepared according to the previously reported procedure (17). Chloro-radicicol A was dissolved in dimethyl sulfoxide as a 10 mM stock solution and further diluted into buffer. Broad profiling of this pharmacophore against a panel of 401 kinases showed it to be a potent and irreversible inhibitor of the following kinases: TAK1, VEGFR, FLT3, GAK, KIT, MEK, MKNK, PDGFR, PRKD, STK36, TGFβ (18). The p38α and β inhibitor, SB203580, were purchased from Calbiochem and used at a concentration of 10 μM.

Immunoblotting and Immunostaining—HEK293 cell lines and PC-3U cells were grown in 10-cm dishes. Forty-eight hours after transfection, cells were starved for 16 h and stimulated with TNF-α, IL-1β, TGFβ, or LPS for the indicated time periods, washed once with ice-cold PBS, and lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% Triton X-100, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 1 mM aprotinin, 1 mM Pefabloc, 1 mM sodium orthovanadate). After centrifugation, supernatants were collected, and protein concentrations were measured by using the BCA protein assay kit (Fisher Scientific). Equal amounts of proteins were used for immunoprecipitations. Samples were subjected to SDS-PAGE in 8% or 10% polyacrylamide gels, followed by blotting onto polyvinylidene difluoride membranes and immunoblotting as described previously (19).

For analysis by immunostaining, PC-3U or RAW264.7 cells were seeded in 6-well plates and grown on coverslips 24 h before transfection. Immunostaining was performed as described previously (16). Photomicrographs were obtained by an Axioskop 2 microscope (Carl Zeiss MicroImaging, Inc.) with a digital camera (C4742-95, Hamamatsu) using a Plan Apochromat ×63/1.40 oil DIC objective lens (Carl Zeiss MicroImaging, Inc.). Photographs were taken at room temperature. Primary images were acquired using the Velocity program. Image memory content was reduced, and brightness contrast was adjusted using Photoshop 6.0 (Adobe).
In Vivo Ubiquitination Assays—HEK293 and RAW264.7 cells were transfected as described in the figure legends, washed once in PBS, scraped in 1 ml PBS, and centrifuged at 400 g for 5 min. Non-covalent protein interactions were dissociated with 1% SDS and boiling for 5–10 min. Samples were diluted in PBS (1:10) containing 0.5% Nonidet P-40, 1 mM aprotinin, and 1 mM Pefabloc. The sample was cleared by centrifugation at 12,000g for 10 min. The supernatants were subjected to immunoprecipitation and followed by immunoblotting as indicated in the figure legends.

ELISA—For analysis of cytokine production, PC-3U or RAW264.7 cells were seeded into 6-well plates 24 h before transfection. After stimulation of cells for 24 h, the supernatant of the cell culture medium was collected. IL-8 or IL-6 secretion into the supernatant was determined by using the ELISA BD OptEIA™ set human IL-8 or mouse IL-6 from BD Biosciences according to the manufacturer’s protocols. ELISA data are plotted in bar graphs representing the mean ± S.E. of at least three independent experiments performed in duplicates unless noted otherwise.

Plasmids—Expression vectors for 3× HA-tagged TAK1, TAK1-K63W, and dN-TRAF6 were gifts from K. Matsumoto (University of Nagoya, Japan). FLAG-tagged TRAF6-C70A was a kind gift from Z. J. Chen (University of Texas Southwestern Medical Center, Dallas, TX). FLAG-ubiquitin was a gift from I. Dikic and K. Haglund (Goethe University, Frankfurt, Germany). 3× HA-tagged TAK1-K34R was generated and described by us previously (4). The Wild-type and NF-κB mutant IL-6 promoter was a gift from D. L. Allen, University of Colorado, Boulder, CO (20).

Luciferase and β-Galactosidase Assays—Cells were transiently transfected with the pNF-κB-Luc-cis reporter plasmid (0.5 μg, Stratagene) containing a 5× NF-κB enhancer element in the absence or presence of expression vectors for TAK1, dN-TRAF6, or pcDNA3. Luciferase activities were determined in triplicate samples with the enhanced luciferase assay kit according to the manufacturer’s protocol (BD Biosciences). The reporter plasmid pCMV-β-Gal (0.2 μg) was transfected in each condition and used as a reference. Normalized promoter activity data are plotted in bar graphs representing the mean ± S.E. of at least three independent experiments performed in triplicate, unless noted otherwise.

Statistical Analysis—Statistical analyses between all groups were performed using the analysis of variance test. Values are expressed as mean ± S.E. of at least three independent experiments, unless noted otherwise. p values of < 0.05 were considered as statistically significant.

RESULTS

Polyubiquitination of TAK1 in TNF-R, IL-1R, and TLR4 Signaling—To investigate whether Lys-63-linked polyubiquitination of TAK1 plays a general role in NF-κB signaling, we used HEK293 cell lines as a model to study different proinflammatory pathways in the same cell type. TNFR signaling was studied in WT HEK293 cells, whereas LPS-induced signaling was investigated in HEK293 cells stably transfected by TLR4 (HEK293-TLR4) and IL-1R signaling in HEK293 stably transfected by IL-1R (HEK293-IL-1R). We performed in vivo ubiquitination immunoprecipitation assays by overexpressing HA-TAK1-WT together with FLAG-ubiquitin and treated cells with TNF-α, LPS, or IL-1β (Fig. 1). TAK1-WT underwent robust polyubiquitination in all three pathways. Although TNF-α-mediated (Fig. 1A) and IL-1β-mediated (C) polyubiquitination of TAK1 were rapidly induced after 5 min, LPS-induced ubiquitination occurred earliest at 30 min (B, in accordance with previous observations) (21).

In conjunction with polyubiquitination of TAK1, phosphorylation of TAK1 was detected after 5 min of IL-1β stimulation, which correlated with phosphorylation and degradation of IκBα (Fig. 1, C and D).

Polyubiquitination of TAK1 Is Dependent on Lys-34 in TNFR-, IL-1R-, and TLR4-mediated Signaling—We have recently shown that TRAF6-mediated Lys-63-linked polyubiquitination of Lys-34 in TAK1 correlates with TAK1 activation (4). Exposure to IL-1β recruits endogenous TAK1 to the TRAF6 complex and activates TAK1, which leads to NF-κB activation (22, 23). To examine the role of the E3 ligase TRAF6 for ubiquitination of TAK1 in the IL-1β signaling pathway, we overexpressed a TRAF6 mutant lacking the E3 activity (TRAF6C70A). TAK1 polyubiquitination occurred after expression of WT TRAF6 but not after expression of TRAF6C70A (Fig. 2A). Furthermore, IL-1β-induced TAK1 phosphorylation was observed only when HAE-TAK1-WT was cotransfected with FLAG-PolyUb and TRAF6-WT (Fig. 2B).
ylation and phosphorylation of IKKα/β was blocked by TRAF6 C70A (Fig. 2B).

To investigate whether polyubiquitination of TAK1 occurs at Lys-34 in other signaling pathways than in the TGFβ signaling pathway, we investigated the effect of transfection of the mutated HA-TAK1 (K34R) (Fig. 3A), LPS- (B), and TNF-α-stimulated (C) cells. We detected a substantial decrease of IL-1β-induced (Fig. 3A), LPS-induced (B), and TNF-α-induced (C) TAK1 polyubiquitination when the K34R mutant TAK1 was overexpressed. These results indicated that polyubiquitination of TAK1 on Lys-34 is induced by different cytokines regulating inflammatory pathways. The association with NEMO and TAK1 was not affected by the K34R mutation (Fig. 3D). Furthermore, polyubiquitination of TAK1 on Lys-34 was found to be required for activation of TAK1 and IKKα/β.

NF-κB Signaling Is Dependent on TAK1-K34-dependent Polyubiquitination in Different Pathways—Previous observations have revealed that TAK1 is crucial for NF-κB activation in the TNFR, IL-1R, and TLR4 signaling pathways (23–26). To determine whether polyubiquitination of TAK1 is required for proinflammatory cytokine- and LPS-induced NF-κB activation, we performed NF-κB luciferase reporter assays. We investigated the role of K34R mutant TAK1 for TNF-α-induced activation of NF-κB in HEK293 cells by

FIGURE 2. Polyubiquitination of TAK1 is dependent on TRAF6 in IL-1R signaling. A, 293-IL-1R cells were transfected with FLAG-TRAF6 WT or FLAG-TRAF6 C70A cDNA. After treatment with 10 ng/ml IL-1β for 5 and 10 min, cell lysates were prepared and immunoprecipitated (IP) with (monoclonal mouse) anti-TAK1, followed by immunoblotting (IB) for anti-ubiquitin. The IP filter was reblotted with (polyclonal rabbit) anti-TAK1. B, total cell lysates were subjected to immunoblotting for p-TAK1 and then reblotted with TAK1 as well as with p-IKKα/β and then reblotted with IKKα/β. Finally, the filter was reblotted with β-actin to demonstrate equal loading of proteins in all lanes.

FIGURE 3. Polyubiquitination of TAK1 is dependent on Lys-34 in the TNFR, IL-1R, and TLR4 signaling pathways. Ubiquitination of TAK1 was studied by transfecting HEK293-IL-1R cells (A), HEK293-TLR4 cells (B), and HEK293 cells (C) with FLAG-ubiquitin, HA-TAK1, or HA-K34R cDNA. Cells were stimulated with 10 ng/ml IL-1β (A), 500 ng/ml LPS (B), or 10 ng/ml TNF-α (C), for different time periods as indicated. An in vivo ubiquitination assay was performed, and lysates were subjected for immunoprecipitation (IP) with anti-HA and immunoblotting (IB) with anti-FLAG. The asterisk indicates a background band in the upper panel of A. The IP filter was reblotted with anti-TAK1. D, HEK293 cells were transfected as indicated in the figure, and IP was performed using anti-Myc antibody, followed by IB for anti-HA. Total cell lysates were subjected to immunoblotting for TAK1 and Myc (to visualize 6myc-NEMO).
transient transfections. Expression of TAK1-K34R prevented NF-κB activation.

A similar result was obtained for TAK1-K34R upon IL-1β and LPS exposure (Figs. 5A and 6A). Moreover, the K34R mutant TAK1 suppressed NF-κB activation dose-dependently in TNF-α (Fig. 4B), IL-1β- (Fig. 5B), and LPS-stimulated (Fig. 6B) cells. As expected (25), coexpression of a kinase-dead form of TAK1 (K63W) significantly down-regulated NF-κB activity in all three pathways (Figs. 4A, 5A, and 6A).

We also investigated the effect of the TAK1 inhibitor chlororadicol A in our assays. By pretreating the cells with 2 μM chlororadicol A, TNF-α, IL-1β-, or LPS-induced activation of NF-κB was efficiently blocked (Figs. 4C, 5C, and 6C). TLR4-transfected HEK293 cells have constitutive NF-κB activation (27, 28), but the HEK293-TLR4 cell line slightly responded to 500 ng/ml LPS stimulation (Fig. 6). Notably, transfection of TAK1-K34R or TAK1-K63W, or treatment with chlororadicol A, inhibited the constitutively active NF-κB in this cell line.

TGFβ induces auto-ubiquitination of TRAF6 and subsequent activation of the TAK1-p38 pathway (4). As previous observations demonstrated, a role of TAK1 in TGFβ-mediated NF-κB signaling (29), we investigated whether polyubiquitination of TAK1 on Lys-34 could affect NF-κB signaling in TGFβ-stimulated HepG2 cells (Fig. 7). A significant decrease in NF-κB activation was found by overexpressing TAK1-K34R, TAK1-K63W, or DN-TRAF6 (Fig. 7A). Furthermore, chlororadicol A suppressed TGFβ-induced activation of NF-κB, although the p38 MAP kinase inhibitor SB203580 had no effect on NF-κB regulation (Fig. 7B). These data show that TRAF6-dependent polyubiquitination of TAK1 on Lys-34 was required for activation of NF-κB in TNF-α, IL-1β, LPS, and TGFβ signaling. In addition, p38 was not needed for the activation of NF-κB by TGFβ.

K34R-mutant TAK1 Prevents NF-κB Activation and Translocation into the Nucleus in Prostate Cancer Cells—It has recently been proposed that prostate cancer is driven by inflammation (30). However, how chronic inflammation can cause prostate cancer development is not completely known yet. The transactivation factor NF-κB plays a central role in inflammation. Further, NF-κB-dependent mechanisms enhance tumor promotion during inflammation. Therefore, we investigated whether TAK1 ubiquitination affects proinflammatory signals in prostate cancer cells. The human prostate cancer cell line (PC-3U) was stimulated with TNF-α, and NF-κB activation was monitored by a NF-κB luciferase reporter assay. In line with our previous results in HEK293 cells, we found that NF-κB activation in PC-3U cells was dependent on polyubiquitination of TAK1 at Lys-34 (Fig. 8A). Nuclear fractionation assays in PC-3U cells revealed that p65 is located in the nucleus after exposure to TNF-α and that this event was enhanced by overexpression of WT-TAK1 (Fig. 8B). In contrast, TAK1-K34R attenuated nuclear localization of p65 after stimulation with TNF-α.

To further analyze the localization of p65, immunofluorescence studies were performed in PC-3U cells (Fig. 8C). WT HA-TAK1 and HA- K34R mutant TAK1 expressing cells were stained with anti-HA and anti-p65 antibodies. Although most of the p65 was present in the cytoplasm in untreated cells, TNF-α-stimulation was found to induce nuclear translocation of p65.

**FIGURE 4.** TNF-α-induced activation of NF-κB is prevented by TAK1-K34R. A, HEK293 cells were transfected with cDNAs for TAK1 K63W or K34R cDNA (0.5 μg) or empty vector (0.5 μg) together with the CMV-β-galactosidase and NF-κB reporter plasmid. Twenty-four hours after transfection, cells were stimulated with 10 ng/ml TNF-α for 6 h. Cells were lysed, and luciferase and β-galactosidase activities were measured as described under “Experimental Procedures.” The activity of the NF-κB reporter was normalized according to β-galactosidase activities in the cells. ***, p < 0.001; *, p < 0.05. Expression levels of mutant TAK1 were analyzed by subjecting the corresponding lysates to immunoblotting (IB) for anti-HA and anti-β-actin. B, HEK293 cells were transfected with different amounts of TAK1-K34R cDNA (0.01, 0.05, and 0.5 μg), and stimulated with TNF-α as indicated in A. C, HEK293 cells were pretreated or not pretreated with 2 μM chloro-radicol A (chlradA) 30 min before stimulation with TNF-α. ***, p < 0.001 in B and C. The data are plotted in bar graphs representing mean ± S.E. of three independent experiments.
in mock- and HA-TAK1-WT-transfected cells. Notably, in PC-3U cells transfected with HA-K34R-mutant TAK1, p65 was mainly localized in the cytoplasm, whereas p65 was localized in the nucleus in ~70% of TNF-α-treated cells transfected with WT HA-TAK1.

One of the key events in the TNF-induced inflammation is the induction of IL-6 and IL-8, which are involved in carcinogenesis (31). Hence, we tested whether IL-6 promoter activation and IL-8 production in PC-3U cells were affected by TAK1 ubiquitination in TNF-α-treated cells (Fig. 8D). TNF-α-induced secretion of IL-8 (Fig. 8E), was enhanced by overexpression of WT TAK1 and attenuated by overexpression of K34R mutant TAK1.

To further evaluate the physiological importance of TAK1 polyubiquitination for its subsequent activation and downstream signaling toward NF-κB and IL-6 secretion, we included the mouse macrophage line RAW264.7 in our study. As shown in Fig. 9A we observed LPS-induced polyubiquitination of TAK1 20 min after stimulation of cells, only in cells transfected with WT-TAK1, but not in cells transfected with K34R mutant TAK1. This result correlated with LPS-induced activation of TAK1 in the WT-TAK1-transfected mouse macrophage RAW264.7 cell line, which was clearly reduced by K34R. IKK is known to be the main activator of NF-κB (6). Hence, we examined the role of TAK1 polyubiquitination on Lys-34 in activation of the IKK complex. Transfection of K34R-mutant TAK1 resulted in an inhibition of IKKα/β phosphorylation in RAW264.7 cells stimulated with LPS. Chloro-radicicol A efficiently inhibited LPS-induced phosphorylation of TAK1, IKKα/β (Fig. 9C), as well as activation of NF-κB in TAK1 overexpressing RAW264.7 cells (D). Transfection of K34R-mutant TAK1 also reduced activation of NF-κB and IL-6 promoter activation/secretion in RAW264.7 cells (Fig. 9, E and F).

DISCUSSION

We have recently shown that the E3 ligase TRAF6 mediates Lys-63-linked polyubiquitination of TAK1 at K34 which is required for activation of TAK1 itself as well as p38 MAP kinase in TGFβ-stimulated cells (4). As the principle of Lys-63-linked ubiquitination in controlling protein-protein interaction and thereby activating kinase cascades are shared by other pathways such as TNF-R, IL-1R and TLR4 signaling, we investigated if Lys-63-linked polyubiquitination of TAK1 is a common molecular mechanism leading to its activation. We found that TAK1 underwent polyubiquitination in response to ligand-induced activation of TNF-R, IL-1R, and TLR4, and that mutation of Lys-34 (K34R) in TAK1 resulted in a loss of TAK1 and IKK activation, which prevented activation and translocation of

FIGURE 5. IL-1β-induced activation of NF-κB is blocked by TAK1-K34R. 293-IL-1R cells were transfected with cDNAs for TAK1-K63W (0.5 μg) or TAK1-K34R (0.5 μg) (A) or with different amounts of K34R (0.01, 0.05, 0.5 μg) (B). The NF-κB luciferase reporter activity was analyzed after stimulation of 293-IL-1R cells with IL-1β 10 ng/ml. The corresponding lysates were subjected to immunoblotting (IB) for anti-HA or β-actin. C, after pretreatment or no pretreatment with 2 μM of chloro-radicicol A (chlradA) for 30 min, cells were stimulated with IL-1β for 6 h. Then cells were lysed, and the NF-κB luciferase reporter assay was performed. D, 293-IL-1R cells were transfected with WT-TAK1 or TAK1-K34R cDNA (0.5 μg). The WT-pIL-6 and mutant p-IL6 NF-κB luciferase reporter activity was analyzed after stimulation of 293-IL-1R cells with IL-1β. ***, p < 0.001; **, p < 0.01; and *, p < 0.05 (A–D). The data are plotted in bar graphs representing mean ± S.E. of three independent experiments.
NF-κB into the nucleus. The polyubiquitination and activation of TAK1 was faster when induced downstream of IL-1R and TNF-R (5–20 min), whereas downstream of TLR4 it was observed after 20–30 min, in accordance with previous reports from different groups (23–25, 32). The observed differences in kinetics of activation of TAK1 in the various signaling pathways, might be explained by the recent publication from Tseng et al. (32), who interestingly demonstrated the translocation of a multiprotein complex consisting of TLR4, TRAF6, and other regulatory proteins in the pathways, from the cell membrane to an endosomal compartment where TAK1 was found to be activated. This chain of events implicated also degradation of another TRAF family member, i.e. TRAF3, which preceded LPS/TLR4-induced activation of TAK1. Future research will be needed to further sort out the precise chain of molecular events in the different pathways related to kinetics and localization of the signaling components within the activated cell.

The Lys-63-linked ubiquitination regulates NF-κB signal transduction by providing target proteins with an ability to engage in interactions with molecules containing ubiquitin binding domains (UBDs) (33). Lys-63-linked autoubiquitination of TRAF6 is critical for activation of TAK1 and IKK, leading to activation of NF-κB (9, 23). However, the detailed molecular mechanisms for activation of TAK1 were previously unknown. In line with the report from Kishida et al. (34), we demonstrate that TRAF6 mediates Lys-63-linked polyubiquitination of TAK1 in the IL-1β signaling pathway, similar to what was demonstrated for TGFB signaling (4). TAK1 polyubiquitination occurs after 5 min of IL-1β stimulation, which correlates with its activation and a rapid phosphorylation and degradation of IκBα. The early and robust polyubiquitination of TAK1 is
consistent with previous findings of rapid TRAF6 autoubiquitination after stimulation by IL-1β (23). In contrast, LPS-induced polyubiquitination of TAK1 occurs later, at 20 min. Consistent with these findings, ubiquitination of TRAF6 was shown to be induced after 30 min by LPS in RAW264.7 cells (10), supporting the possibility that TRAF6 is also the ubiquitin ligase for TAK1 in the TLR4 signaling pathway. In TNF-α signaling, phospho-
ylated TRAF2 undergoes Lys-63-polyubiquitination, thereby recruiting the TAK1 binding proteins TAB2 and TAB3 to bridge the TAK1 complex to IKKα/β (11). TAK1 is also recruited to the TNFR1 by TRAF2-dependent polyubiquitination of receptor-interacting protein 1 (RIP1), resulting in activation of the IKK catalytic subunits (35, 36). However, the E3 ligase required for TAK1 polyubiquitination in TNF-α signaling remains to be elucidated. A possible ubiquitin ligase mediating TAK1 polyubiquitination in TNF-α signaling is TRAF2, as it contains, like most other TRAFs, a RING domain ligase that likely functions as an E3 (37). Another ubiquitin ligase that is recruited to the TNFR signalosome is inhibitor of apoptosis protein 1 (c-IAP1), which was shown to mediate Lys-63-linked polyubiquitination of NEMO (38).

We previously reported that TGFβ-induced Lys-63-linked polyubiquitination of TAK1 is crucial for its activation and subsequent activation of p38 MAP kinase and induction of apoptosis (4). Here we report that TGFβ induces NF-κB signaling by TRAF6-induced Lys-63-linked polyubiquitinated TAK1 in hepatoma cells (HepG2) independent of p38. Thus, TGFβ induces a prosurvival pathway via NF-κB in hepatocellular carcinoma cells, in parallel to proapoptotic pathways via JNK and p38 (29). An interesting topic for future studies is to explore the mechanism that regulates the balance between the pro- and antiapoptotic pathways.
Polyubiquitination of TAK1 was found to be correlated with its activation, but Lys-63-linked polyubiquitin chains occur also at many other signaling components of the NF-κB pathway, including TAB2, TRAF2, TRAF6, RIP1, and NEMO. In addition, ubiquitin binding domains are found in the TAK1 adapter proteins TAB2, TAB3, and NEMO (3). Multiple polyubiquitin-ubiquitin binding domain interactions may build a network that stabilizes formation of receptor complexes and facilitates correct positioning and complex formation of TAK1 and IKK with the receptors. Another possibility is that Lys-63-linked polyubiquitination of TAK1 directly activates TAK1. The two possibilities do not need to be mutually exclusive. Consistent with the second possibility, deletion of the first 22 amino acids of TAK1 enhances its kinase activity (2), suggesting that the N-terminal region of TAK1 may have an inhibitory effect on its kinase activity. Supporting this possibility, we found that the...
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interaction between TAK1 and TAB2 was reduced when cells were transiently transfected with the K63R mutant ubiquitin to suppress Lys-63-linked polyubiquitination, compared with control cells (4). It is also possible that Lys-63-linked polyubiquitination on TAK1 induces a conformational change that allows TAK1 autophosphorylation of its activation loop (39). Hence, the IKK complex is activated leading to NF-κB-induced transcription of proinflammatory cytokines.

TAK1 polyubiquitination and activation is thus a relatively rapid and transient signaling process that is terminated by the deubiquitinating enzyme CYLD, which removes its ubiquitin chains and inhibits activation of TAK1 (3). In addition, TAK1 activation is counteracted by protein phosphatase 6 by dephosphorylating Thr-187 in the activation loop of TAK1 (40). The underlying molecular mechanisms to explain in detail the differences in kinetics for TAK1 polyubiquitination and subsequent activation in various signaling pathways as observed in our study, as well as in reports from other groups remains to be further explored (10).

During the preparation of this study, two groups reported the identification of Lys-63-dependent polyubiquitination of TAK1 (21, 41). Yamazaki et al. (21) identified Lys-209 as the acceptor for Lys-63-linked ubiquitin chains in response to IL-1 (21). On the other hand, Fan et al. (41) reported that polyubiquitination of TAK1 at Lys-158 is necessary for NF-κB activation in response to IL-1 and TNF-α. Surprisingly, each group tested a number of lysine mutants and excluded the lysine residue at the position suggested by the other group, as well as Lys-34, which was identified by us. Thus, it is possible that there are multiple acceptor sites for Lys-63-linked ubiquitin chains in TAK1. However, it is necessary to verify that TAK1 mutants are correctly folded so that they are not inactive for reasons that do not depend on ubiquitination. As the K34R mutant still interacts with TRAF6 and TGFβ type I receptor, this mutation at least does not impair the overall functional conformation of TAK1 (4).

We were able to efficiently block NF-κB activation with the K34R TAK1 mutant in different cell systems. However, the K34R mutant appeared to be less efficient in inhibiting TNF-α-induced mRNA expression of IL-6 and IL-8, as well as IL-8 secretion by HEK293 and PC3U cells, suggesting that other pathways are also involved in regulation of IL-6 and IL-8 expression. Indeed, the IL-6 promoter is regulated by NF-κB in cooperation with other transcription factors as in prostate cancer cells, transcriptional binding sites for NF-κB, cAMP response element binding protein, C/EBP, and AP-1 have been identified within the IL-6 promoter region (42).

Another possible explanation why the K34R mutant did not completely inhibit activation of its downstream targets could be explained by the presence of endogenous TAK1 in the different cell model systems used in our study.

We found that Lys-63-linked polyubiquitin chains on TAK1 were critical for regulation of NF-κB signaling in prostate cancer cells as well as in mouse macrophages (RAW264.7 line). inflammation is a risk factor for prostate cancer and depends on the production of cytokines in response to tissue damage or the presence of stimuli that induce cellular stress. In the tumor microenvironment, cytokines are secreted not only by the tumor-infiltrating immune cells, but also by the tumor cells themselves. NF-κB-dependent mechanisms have been implicated to be responsible for the enhancement of tumor promotion by inflammation (43, 44). Thus, focusing on TNF-α-mediated NF-κB activation by TAK1, we observed that TAK1 polyubiquitination was crucial for appropriate NF-κB activation and NF-κB-induced gene expression of IL-6 and IL-8 in prostate cancer cells. Although constitutive activation of NF-κB in PC-3 cells was reported before, we found a TNF-α-induced NF-κB translocation that started after 1 h and was sustained over several hours in prostate cancer cells.4 PC-3-milXβ cells that stably express a mutant of IκB lack the capacity to invade and activate bone resorption in conjunction with down-regulation of IL-6 (45). However, the underlying pathways regulating the antiapoptotic effects of NF-κB in prostate cancer cells are not well understood.

Our observation that LPS/TLR4-induced activation of the NF-κB pathway in mouse macrophages (RAW264.7 line) resulted in IL-6 promoter activation and IL-6 secretion is interesting, as in prostate tumors infiltrated with macrophages, this event has been demonstrated to be correlated with treatment resistance as well as tumor progression (46). Therefore, it might be of interest to prevent the vicious tumor-macrophage-related cell communication to prevent tumor progression (6, 47, 48).

FIGURE 9. NF-κB activation and IL-6 are induced downstream of polyubiquitination of TAK1 at Lys-34 in the mouse macrophage cell line RAW264.7. RAW264.7 cells were transfected with TAK1-WT or TAK1-K34R cDNA. A, cells were stimulated with LPS as indicated after 48 h of transfection. Cell lysates were prepared and immunoprecipitated (IP) with anti-HA, followed by immunoblotting (IB) for ubiquitin (P4D1). The IP filter was reblotted with anti-TAK1. Total cell lysates were subjected to immunoblotting for p-TAK1 and reblotted with TAK1, as well as p-IκKα/β and HA, and reblotted with β-actin. B, RAW264.7 cells were grown on coverslips and transfected with HA-TAK1-WT or HA-TAK1-K34R. After 48 h, cells were exposed to LPS for 0.5 h. After fixation with 4% formaldehyde and permeabilization with 0.5% Triton X-100, cells were labeled with antibodies as indicated (green, anti-HA; red, anti-p65). Nuclear staining was performed by staining with DAPI (blue), and cells were labeled with secondary antibody conjugated to Alexa Fluor 488 or TRITC. Nuclear p65 was determined in WT and K34R transfected cells by counting cells stained with anti-HA. Shown is a representative experiment of two experiments. C, RAW264.7 cells cultured with LPS as indicated in the presence or absence of pretreatment with 2 μM of chlororadical A (chloroad) for 30 min. Cell lysates were prepared and subjected to immunoblotting for p-TAK1 and reblotted for TAK1 and p-IκKα/β. D, RAW264.7 cells were transfected with WT-TAK1 or TAK1-K34R cDNA (0.5 μg). After pretreatment or no pretreatment with 2 μM of chloroad for 30 min, cells were stimulated with LPS for 6 h, and NF-κB luciferase reporter activity was analyzed as described under “Experimental Procedures.” E, the WT-pI1-6 and mutant NF-κB p-I1-6 luciferase reporter activity was analyzed after stimulation of RAW264.7 cells transfected with WT-TAK1 or TAK1-K34R cDNA (0.5 μg) and treatment with LPS. F, the supernatant of the culture medium was collected, and the amount of IL-6 production was analyzed by ELISA. The data are plotted in bar graphs representing mean ± S.E. of three independent experiments. Analysis of variance was used to calculate significant differences between all groups. ***p < 0.001; **p < 0.01; *p < 0.05 (D–F).
In conclusion, our study demonstrates a pivotal role of TAK1 polyubiquitination in three different pathways, including TNFR, IL-1R, and TLR4 signaling. Lys-63-linked polyubiquitination of TAK1 at Lys-34 is essential for downstream signaling to NF-κB-mediated target gene expression in both cancer and immune cells. Moreover, Lys-63-linked polyubiquitination of TAK1 could be identified as a crucial factor in maintaining the anti-apoptotic effects of NF-κB in prostate cancer cells. These findings are of importance for the understanding of the mechanism of activation of NF-κB in inflammation and may aid in the development of new therapeutic strategies to treat chronic inflammation and cancer.

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