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TNFα- and IKKβ-mediated TANK/I-TRAF phosphorylation: implications for interaction with NEMO/IKKγ and NF-κB activation

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Pro-inflammatory cytokines trigger signalling cascades leading to NF-κB (nuclear factor-κB)-dependent gene expression through IKK [IκB (inhibitory κB) kinase]-dependent phosphorylation and subsequent degradation of the IκB proteins and via induced phosphorylation of p65. These signalling pathways rely on sequentially activated kinases which are assembled by essential and non-enzymatic scaffold proteins into functional complexes. Here, we show that the pro-inflammatory cytokine TNFα (tumour necrosis factor α) promotes TANK [TRAF (TNF receptor-associated factor) family member associated NF-κB activator] recruitment to the IKK complex via a newly characterized C-terminal zinc finger. Moreover, we show that TANK is phosphorylated by IKKβ upon TNFα stimulation and that this modification negatively regulates TANK binding to NEMO (NF-κB essential modulator). Interestingly, reduced TANK expression by RNA interference attenuates TNFα-mediated induction of a subset of NF-κB target genes through decreased p65 transactivation potential. Therefore the scaffold protein TANK is required for the cellular response to TNFα by connecting upstream signalling molecules to the IKKs and p65, and its subsequent IKKβ-mediated phosphorylation may be a mechanism to terminate the TANK-dependent wave of NF-κB activation.

Key words: nuclear factor κB (NF-κB), p65, tumour necrosis factor (TNF), TNF receptor-associated factor (TRAF), TRAF family member associated NF-κB activator (TANK), RNA interference.

INTRODUCTION

TNFα (tumour necrosis factor α) is a pro-inflammatory cytokine that plays a critical role in a variety of cellular events such as apoptosis, cell proliferation, differentiation and septic shock [1]. Most of the TNFα biological effects occur through binding to its cellular TNFR1 (TNF receptor 1), which triggers several signalling cascades that ultimately cause NF-κB (nuclear factor κB) and AP-1 (activating protein-1) activation. In the case of NF-κB activation, this signalling pathway leads to the IKK [IκB (inhibitory κB) kinase] complex-mediated phosphorylation and degradation of IκBα [2,3]. NF-κB subsequently translocates into the nucleus and induces a variety of target genes. Such a cascade involves sequentially activated kinases that are assembled into functional complexes by non-enzymatic scaffold proteins. They include TRADD (TNFR1-associated death domain protein), which is rapidly recruited to the TNFR1 upon TNFα stimulation [4], and TRAF-2 (TNFR-associated factor 2), which is required for the subsequent recruitment of the IKK complex to the TNFR1 signalosome [5]. The IKK complex also includes a scaffold protein named NEMO (NF-κB essential modulator)/IKKγ (hereinafter referred to as NEMO) [6,7], which is essential for NF-κB activation as demonstrated by the inability of any NEMO-deficient cell to respond to many NF-κB-activating stimuli including TNFα [7]. Although this pathway has been extensively investigated, important issues remain unclear, for example the identity of the TNFα-activated kinase that triggers IKK phosphorylation and subsequent activation *in vivo*. Multiple candidates, including TAK1 [TGF-β (transforming growth factor β)-activated kinase 1] and MEKK3 {MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] kinase 3}, may fulfil this function [8–11]. Also, a variety of phosphorylations that target the scaffold proteins such as NEMO have been described but their significance *in vivo* is still unclear [12–14].

Even if the IKK-mediated phosphorylation and subsequent IκBα degradation is the most important mechanism that leads to NF-κB activation, optimal target gene expression also requires phosphorylation of the NF-κB protein p65 on multiple sites. These phosphorylations critically regulate p65's ability to recruit histone acetyltransferases such as CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 to activate transcription [15]. Moreover, TNFα-induced gene expression also requires an IKKα-mediated histone H3 phosphorylation in the nucleus [16,17]. This latter finding highlights the critical role of IKKα/IKKβ-mediated phosphorylations of other substrates besides IκBα for an optimal NF-κB activation. In this context, NEMO and p65 have also been described as IKKα/IKKβ

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; HEK-293 cell, human embryonic kidney 293 cell; IκB, inhibitory κB; IKK, IκB kinase; IL, interleukin; IRF, interferon regulatory factor; KO, knock-out; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; MEKK3, MEK kinase 3; NAK, NF-κB-activating kinase; NF-κB, nuclear factor κB; NEMO, NF-κB essential modulator; RIP1, receptor interacting protein 1; RNAi, RNA interference; RSV, Rous sarcoma virus; SAPK, stress-activated protein kinase; siRNA, small interfering RNA; TGF-β, transforming growth factor β; TAK1, TGF-β-activated kinase 1; TNF, tumour necrosis factor; TNFR, TNF receptor; TRAF, TNFR-associated factor; I-TRAF, TRAF-interacting protein; TANK, TRAF family member associated NF-κB activator; TBK1, TANK-binding kinase 1 (also known as NAK and T2K); TLR, Toll-like receptor.

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substrates upon TNFα stimulation [12,18] and it is likely that additional but still unidentified candidates are targeted by this kinase complex as well.

Besides those mentioned above, other scaffold proteins have been identified in TNFα-mediated NF-κB activation but their role remains unclear so far. Among them, TANK (TRAF family member associated NF-κB activator)/I-TRAF (TRAF-interacting protein) has been described as a TRAF-2-interacting molecule that positively regulates NF-κB [19] through an interaction with IKKε/IKK-i (inducible IKK) [20] and TBK1 (TANK-binding kinase 1) [21]. TANK/I-TRAF (hereinafter referred to as TANK) also negatively regulates NF-κB through its C-terminal domain but the underlying mechanism remains unclear [19,22,23]. Based on transfection experiments, it is believed that TANK binds and sequesters TRAF-2 in the cytoplasm, thus preventing its recruitment to the TNFR1 [23], but these results require validation under physiological conditions.

We previously demonstrated that the scaffold protein TANK is connected to the IKK complex through binding to NEMO [24]. Here, we show that TANK is recruited to the IKK complex upon TNFα stimulation via a newly described zinc finger motif and demonstrate that TANK is required for TNFα-mediated NF-κB activation and gene expression of selected target genes. Moreover, we show that TANK is phosphorylated by IKKβ in TNFα-stimulated cells and this modification attenuates TANK's ability to associate with NEMO. Therefore we suggest a model where TANK positively regulates NF-κB activation by connecting upstream signalling molecules such as TBK1 to the IKK complex and p65. This wave of NF-κB activation may subsequently be terminated through an IKKβ-dependent TANK phosphorylation mechanism.

MATERIALS AND METHODS

Cell culture and biological reagents

HEK-293 cells (human embryonic kidney 293 cells) and HeLa cells were maintained as described in [25,26]. Wild-type immortalized MEFs (mouse embryonic fibroblasts) as well as IKKα KO (knock-out), IKKβ KO, IKKα/IKKβ double KO and TRAF-2 KO immortalized fibroblasts were gifts from Dr Inder Verma (The Salk Institute, La Jolla, San Diego, CA, U.S.A.) and Dr Tak Mak (Ontario Cancer Institute, University of Toronto, Toronto, Canada) respectively. They were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with fetal bovine serum and antibiotics. The Jurkat cells deficient for RIP1 (receptor interacting protein 1) expression were previously described [27] and were maintained in RPMI 1640 medium supplemented with fetal bovine serum and antibiotics, as were the wild-type Jurkat cells. The TNFR1 and TNFR2 KO MEFs were maintained in DMEM/F12 medium supplemented with 15 % (v/v) serum and antibiotics.

Polyclonal anti-human TANK rabbit antibodies were previously described [24]. Anti-IκBα, anti-NEMO, anti-TRAF-2, anti-Tpl2/Cot, anti-CK2, anti-HA (haemagglutinin) and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) as were anti-HA and anti-IKKα beads. Anti-FLAG antibodies and beads were purchased from Sigma (St. Louis, MO, U.S.A.). Monoclonal anti-IKKε was from Imgenex (San Diego, CA, U.S.A.), whereas anti-IKKα and anti-RIP1 antibodies were from Pharmingen (San Diego, CA, U.S.A.). Anti-TBK1/NAK (NF-κB-activating kinase) and anti-MEKK3 antibodies were from Abcam (Cambridge, U.K.) and Upstate (Dundee, U.K.) respectively. Recombinant TNFα was obtained from Roche Biochemicals (Mannheim, Germany).

Human FLAG–TANK and truncation mutants of TANK were previously described, as were FLAG–TANKΔIKKε, HA–NEMO, HA–IKKβ and the IKKε–Myc construct [24]. FLAG–TANKΔC50, ΔC35, ΔC20, ΔC15 and ΔC10 constructs were generated by PCR using the FLAG–TANK construct as template. Four distinct GST (glutathione S-transferase)–TANK constructs were subcloned by PCR (GST–TANK amino acids 141–281, GST–TANK amino acid 282 to stop codon, GST–TANK amino acid 282–374 and GST–TANK amino acid 374 to stop codon) into the pGEX-6P3 (Amersham Biosciences, Piscataway, NJ, U.S.A.), and the corresponding fusion proteins, produced and purified by standard methods, were used as substrates for the kinase assays (see below). For site-directed mutagenesis, FLAG–TANK or GST–TANK was used as template to generate TANK mutants, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), by the method provided by the manufacturer. All the resulting TANK mutants were sequenced.

The GAL4 reporter plasmid harbours a GAL4-responsive sequence cloned upstream of a luciferase gene (ClonTech, Palo Alto, CA, U.S.A.), whereas the GAL4–p65 (amino acids 286–551) encodes the indicated p65 functional regions fused to the GAL4 DNA-binding domain and was generously provided by Dr T. Okamoto (Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan).

Immunoprecipitations, phospholabelling and kinase assays

Immunoprecipitations involving overexpressed proteins were performed in HEK-293 cells as previously described [28].

For endogenous immunoprecipitations of the IKK complex, HEK-293 cells (1×10^7) were left untreated or stimulated with TNFα (100 units/ml) for the indicated periods of time and subsequently lysed in the lysis buffer [25 mM Hepes, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 10 % glycerol, 5 mM EDTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM NaF, 25 mM β-glycerophosphate and 'Complete Protease Inhibitor' mixture (Roche Molecular Biochemicals)]. Anti-IKKα immunoprecipitations were carried out using the anti-IKKα beads incubated with the cell lysates overnight at 4 °C. Anti-HA immunoprecipitations (negative control) were performed similarly. The immunoprecipitates were subsequently subjected to Western-blot analyses.

For endogenous IKK activity, anti-NEMO immunoprecipitates were used in kinase assays using 1 μg of substrate (GST–TANK or GST–IκBα) as described in [25]. Phosphorylated TANK or IκBα proteins were detected by autoradiography. For ectopically expressed IKK activity, cells were transfected with the FLAG–TANK or mutant TANK constructs along with HA–IKKβ and/or HA–NEMO, and anti-FLAG immunoprecipitations were performed, as described in [28]. Immunoprecipitated complexes were subsequently used as substrates for the kinase assays, by the method described above.

For the phospholabelling experiments, HEK-293 cells or HeLa cells were incubated with DMEM without phosphate for 48 h followed by a 2 h incubation with [^{32}P]P_i. Cells were subsequently left unstimulated or treated with TNFα (1000 units/ml) for the indicated periods of time. Cell extracts were subjected to immunoprecipitations with an anti-TANK antibody and overnight incubation with Protein A–agarose. The immunoprecipitates were subjected to SDS/PAGE and autoradiography.

RNAi (RNA interference), luciferase assays and real-time PCR

For RNAi, decreased TANK expression was obtained by transfecting SMART POOL of TANK RNAi (Dharmacon, Lafayette,

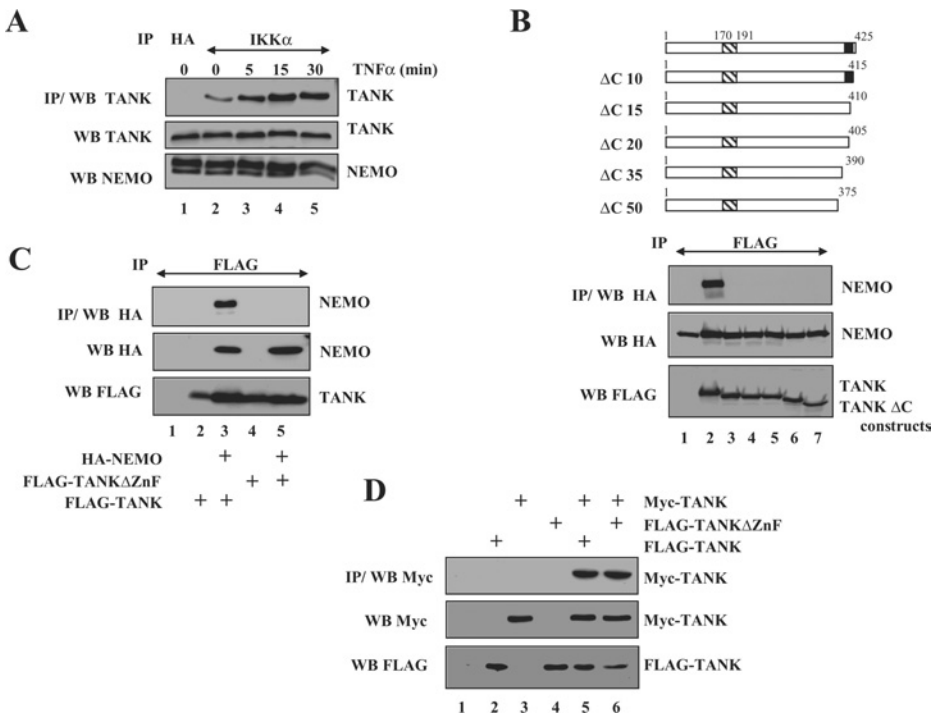

Figure 1 TANK is recruited to the IKK complex upon TNFα stimulation and its zinc finger is required for interaction with NEMO

(**A**) TNFα triggers the recruitment of TANK to the IKK complex. HEK-293 cells were left untreated or stimulated with TNFα (100 units/ml) for 5–30 min and cell extracts were subjected to anti-IKKα immunoprecipitations followed by anti-TANK Western-blot analyses (top panel). The presence of TANK and NEMO in the cell extracts is illustrated by Western blots using the corresponding antibodies (second and third panels from the top respectively). (**B**, **C**) TANK interacts with NEMO through its C-terminal zinc finger. (**B**) The C-terminal domain of TANK is required for binding to NEMO. Upper panels: schematic representation of the TANK constructs used in the co-immunoprecipitation experiments. Lower panels: HEK-293 cells were transfected with HA–NEMO alone (lane 1) or in combination with FLAG–TANK or FLAG–TANKΔC10, -ΔC15, -ΔC20, -ΔC35 or -ΔC50 (lanes 2–7 respectively). Cell extracts were subjected to anti-FLAG immunoprecipitations followed by anti-HA Western-blot analyses (top panel). The presence of HA–NEMO and the FLAG-tagged TANK proteins in the cell extracts is illustrated in the second and third panels from the top respectively. (**C**) The TANK C-terminal zinc finger motif is required for interaction with NEMO. HEK-293 cells were transfected with pcDNA3 (lane 1) or with FLAG–TANK (lanes 2 and 3), FLAG–TANKΔZnF (lanes 4 and 5) and HA–NEMO (lanes 3 and 5), as indicated and cell extracts were subjected to an anti-FLAG immunoprecipitation followed by anti-HA Western-blot analyses (top panel). Cell extracts were subjected to anti-HA and -FLAG Western blots as well (middle and bottom panels respectively). (**D**) The TANK C-terminal zinc finger motif is dispensable for TANK dimerization. Cells were transfected with empty pcDNA3 (lane 1), FLAG–TANK or FLAG–TANKΔZnF alone (lanes 2 and 4 respectively), Myc–TANK alone (lane 3) or a combination of Myc–TANK with FLAG–TANK or FLAG–TANKΔZnF (lanes 5 and 6 respectively), as indicated. Anti-FLAG immunoprecipitations followed by anti-Myc Western-blot analyses were carried out (top panel), whereas anti-FLAG and -Myc Western blots were performed on the cell lysates (bottom panels). IP, immunoprecipitation; WB, Western blot.

CO, U.S.A.) using the Oligofectamine reagent by the method provided by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.).

Luciferase assays were performed in HEK-293 cells using 1 μg of the Ig–κB–luciferase or 100 ng of the GAL4 reporter plasmid and with expression plasmids as indicated by a method previously described [28]. For the reporter assays using the GAL4 DNA-responsive element-containing plasmid, luciferase activities were normalized with *Renilla* luciferase activities, whereas reactions were normalized with β-galactosidase activities for the assays using Ig–κB–luciferase. Real-time PCRs were performed as previously described [28].

RESULTS

TANK is recruited to the IKK complex upon TNFα stimulation and binds NEMO through a newly characterized C-terminal zinc finger motif

We previously showed that TANK is connected to the IKK complex through an interaction with NEMO but the physiological signal that triggers this interaction had not been identified [24]. Because TANK is a TRAF-2-binding protein, an adaptor that plays a role in the TNFα signalling pathway, we investigated whether TNFα stimulation triggers the recruitment of TANK to the IKK complex. HEK-293 cells were left untreated or stimulated with this cytokine and anti-IKKα immunoprecipitations followed by anti-TANK Western blots were performed. TANK interacted with the IKK complex in unstimulated cells and this interaction was enhanced after 5–15 min of TNFα stimulation (Figure 1A, top panel, compare lane 2 with lanes 3–5). Therefore this pro-inflammatory cytokine is a physiological signal that triggers the recruitment of TANK to the IKK complex.

We previously demonstrated that TANK harbours two NEMO-interacting domains, namely an N-terminal region located upstream of the TRAF-interacting site and a 178 amino acid-long C-terminal region [24], which was also required for interaction with IKKβ (A. Chariot and U. Siebenlist, unpublished work). To more precisely define this latter C-terminal NEMO-interacting domain, we tested the ability of additional TANK mutants lacking various lengths of the C-terminal sequence to interact with NEMO by co-immunoprecipitations and observed that any C-terminal deletion of at least 10 amino acids of TANK impaired the association with NEMO (Figure 1B, lanes 3–7). The C-terminal domain harbours a newly identified C_2H_2 zinc finger motif (amino acids 396–420) that includes two cysteine residues (Cys^{396} and Cys^{399}). Therefore we investigated whether such a

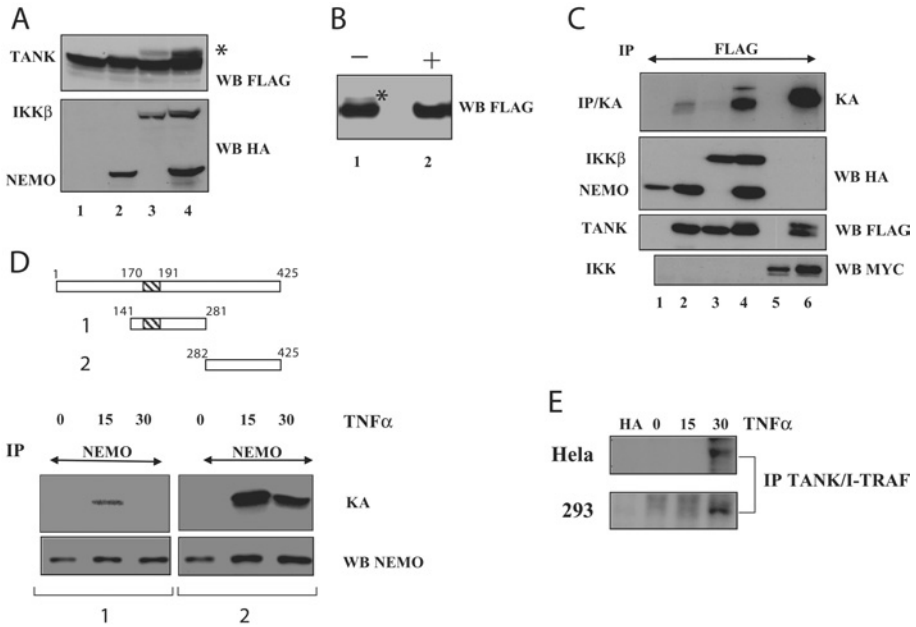

Figure 2 TANK is phosphorylated by the IKK complex in response to TNFα

(**A**) Co-expression of TANK with NEMO and/or IKKβ leads to slower migrating forms of TANK, indicated by an asterisk. HEK-293 cells were transfected with FLAG–TANK alone (lane 1) or in combination with HA–NEMO (lane 2) or HA–IKKβ or both of them (lanes 3 and 4 respectively). Anti-FLAG and -HA Western blots were performed on cell lysates (upper and lower panels respectively). (**B**) Alkaline phosphatase treatment of the cell extracts derived from HEK-293 cells transfected with FLAG–TANK, HA–IKKβ and HA–NEMO inhibits the slower migrating forms of TANK. Cell extracts were left untreated (−) or incubated (+) with alkaline phosphatase for 1 h at 37 °C and anti-FLAG Western-blot analyses were performed. (**C**) IKK-mediated phosphorylation of TANK *in vitro*. HEK-293 cells were transfected with HA–NEMO alone (lane 1) or with FLAG–TANK with either HA–NEMO or HA–IKKβ (lanes 2 or 3 respectively) or with both of them (lane 4). As controls, HEK-293 cells were transfected with IKKε–Myc alone (negative control, lane 5) or with both IKKε–Myc and FLAG–TANK (positive control, lane 6). Anti-FLAG immunoprecipitates were subjected to an *in vitro* kinase assay (top panel). Anti-HA, anti-FLAG and anti-Myc Western-blot analyses were performed on cell extracts (second, third and bottom panels respectively). (**D**) TNFα-mediated phosphorylation of TANK on its C-terminal domain. Upper panel: schematic representation of the GST–TANK fusion proteins used as substrates in the kinase assays. Lower panels: HEK-293 cells were left untreated or stimulated with TNFα for the indicated times and anti-NEMO immunoprecipitations were performed. The GST–TANK fusion proteins 1 or 2 were used as substrates for the kinase assays (left and right panels respectively). An anti-NEMO Western blot performed on the cell extracts is illustrated (bottom panel). (**E**) TNFα-mediated phosphorylation of endogenous TANK in HeLa or HEK-293 cells (upper and lower panels respectively). Both cell lines were cultured in the presence of $[^{32}P]P_i$ and subsequently stimulated with TNFα for the indicated periods of time. Anti-TANK immunoprecipitations were carried out and the immunoprecipitates were migrated on an SDS/PAGE followed by autoradiography. IP, immunoprecipitation; KA, kinase assay; WB, Western blot.

motif is required for the interaction with NEMO by testing the ability of a TANK mutant where both cysteine residues were replaced by alanines ('TANKΔZnF') to bind to this docking protein. Whereas wild-type FLAG–TANK bound HA–NEMO, TANKΔZnF did not (Figure 1C, top panel, lanes 3 and 5 respectively). Moreover, as TANK oligomerizes through its C-terminal domain [29], we investigated whether this association also requires an intact C_2H_2 zinc finger motif. Both FLAG-tagged wild-type and TANKΔZnF interacted with Myc-tagged TANK (Figure 1D, top panel, lanes 5 and 6 respectively), suggesting that the C_2H_2 zinc finger motif is dispensable for TANK oligomerization. Therefore the C-terminal domain of TANK harbours a zinc finger motif required for interaction with the IKK complex but dispensable for oligomerization and for binding to IKKε [24].

TANK is a substrate of the IKK complex

In the process of characterizing the interaction between TANK and the members of the IKK complex, we noticed that co-expression of TANK and the kinase IKKβ led to slower migrating forms (Figure 2A, lane 3). These slower migrating forms of TANK were more intense when NEMO was overexpressed as well, thus suggesting that TANK is phosphorylated by a NEMO-interacting kinase, potentially the IKK complex itself. Indeed, alkaline phosphatase treatment of such an extract caused the total disappearance of the slower migrating forms of TANK (Figure 2B, compare lanes 1 and 2), therefore suggesting that TANK is phosphorylated. Among the NEMO-interacting kinases, IKKα and IKKβ are the catalytic subunits of the IKK complex and are constitutively associated with NEMO. To determine whether TANK is phosphorylated by IKKβ, FLAG–TANK was transiently co-expressed in HEK-293 cells either with NEMO and/or IKKβ. Because IKKε has been described as a TANK-phosphorylating kinase [20], IKKε–Myc was co-expressed with FLAG–TANK as a positive control. *In vitro* kinase assays using anti-FLAG immunoprecipitates as substrates were performed. As expected, IKKε strongly phosphorylated TANK (Figure 2C, top panel, lane 6). TANK was weakly phosphorylated when co-expressed with NEMO but a stronger signal was detected when TANK was co-expressed with both NEMO and IKKβ (Figure 2C, top panel, lane 4). These results therefore suggest that TANK is phosphorylated by two distinct kinases, namely IKKε and IKKβ, *in vitro*. To explore whether this phosphorylation occurs in cells stimulated with a physiological stimulus known to trigger IKK activation, HEK-293 cells were treated or not with TNFα for 15 or 30 min and anti-NEMO immunoprecipitates were subjected to kinase assays using two distinct purified GST–TANK fusion proteins as substrate. The first one harboured the TRAF-2-interacting domain and the IKKε-phosphorylated domain [24] while the second one harboured the very C-terminal end of TANK, from amino acids 282 to 425 (Figure 2D). A TNFα-dependent phosphorylation of TANK was barely detectable with the first GST fusion protein, while the substrate harbouring the C-terminal end of TANK was intensively phosphorylated upon TNFα stimulation (Figure 2D, upper panels, compare left and right panels).

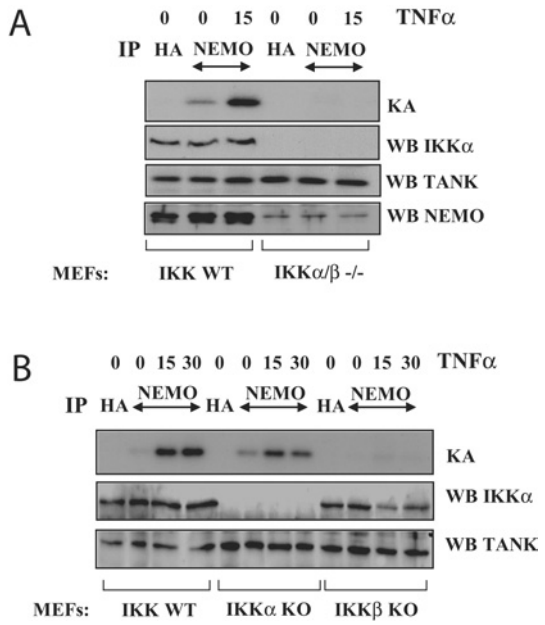

Figure 3 TNFα-mediated phosphorylation of TANK requires IKKβ

(**A**) TNFα-mediated phosphorylation of TANK requires the IKK complex. WT (wild-type) or IKKα/IKKβ double KO MEFs were either untreated or stimulated for 15 min with TNFα. Anti-NEMO or -HA (negative control) immunoprecipitates (IP) were incubated with the GST–TANK used as substrate for a kinase assay (KA; top panel). Anti-IKKα, -TANK and -NEMO Western-blot (WB) analyses were performed on the cell lysates (second, third and bottom panels respectively). (**B**) IKKβ but not IKKα is required for TNFα-mediated TANK phosphorylation. WT, IKKα or IKKβ KO MEFs were used as cellular models to assess TNFα-mediated TANK phosphorylation as described above.

Therefore all subsequent kinase assays addressing TANK phosphorylation were carried out with this latter substrate. These results suggest that TANK is a substrate of the IKK complex, at least *in vitro*. To investigate whether TANK is phosphorylated *in vivo*, HeLa or HEK-293 cells were incubated with [^{32}P]P_i, untreated or stimulated with TNFα and cell extracts were subjected to anti-TANK immunoprecipitation. Phosphorylated TANK was detected from cells treated for 30 min with TNFα (Figure 2E). Taken together, these results demonstrate that TANK is phosphorylated *in vitro* as well as *in vivo* upon TNFα stimulation.

TNFα-mediated phosphorylation of TANK occurs through the TNFR1 and requires IKKβ and RIP1

To determine whether IKKα/IKKβ is required for the TNFα-induced TANK phosphorylation, a similar kinase assay using the GST–TANK fusion protein as substrate was performed in wild-type or IKKα/IKKβ double KO MEFs. Again, a TNFα-dependent TANK phosphorylation was detected in wild-type MEF cells but not in IKKα/IKKβ double KO cells (Figure 3A, top panel). Similar experiments were performed using IKKα or IKKβ KO MEF cells. Whereas TANK phosphorylation still occurred in the TNFα-stimulated IKKα KO cells, such phosphorylation was not detectable in IKKβ KO cells (Figure 3B, top panel), suggesting that IKKβ is the subunit of the IKK complex that phosphorylates TANK. As additional evidence for IKKβ being the TNFα-activated TANK kinase, we addressed TNFα-mediated TANK phosphorylation in cells where the IKK complex is inactivated by BAY 11-7085 [30], an IKK inhibitor. Whereas phosphorylated forms of TANK were detected in TNFα-stimulated HeLa cells by Western-blot analyses, these slower migrating

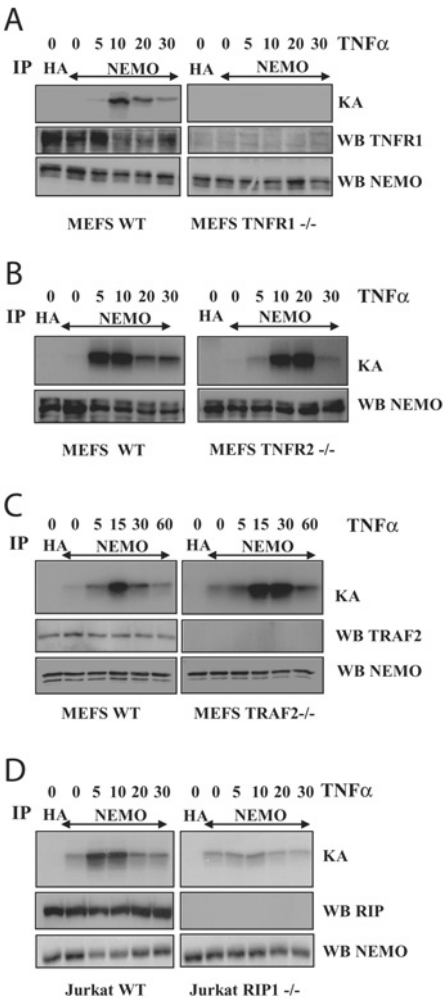

Figure 4 TNFα-mediated phosphorylation of TANK requires RIP1 and the TNFR1 but not the TNFR2, TRAF-2 and IKKα

(**A**, **B**) The TNFR1 but not the TNFR2 is required for the TNFα-mediated TANK phosphorylation. Wild-type (WT) (**A**, **B**), TNFR1 KO (**A**) or TNFR2 KO (**B**) MEF cells were left untreated or stimulated with TNFα for the indicated periods of time and *in vitro* kinase assays (KA) were carried out. Anti-NEMO (**A**, **B**) and -TNFR1 (**A**) Western-blot (WB) analyses were performed using the cell extracts (panels below the kinase assays). (**C**) IKK-mediated phosphorylation of TANK in TRAF-2 KO MEF cells. MEF cells lacking TRAF-2 were left untreated or stimulated for 5, 15, 30 or 60 min by TNFα and *in vitro* kinase assay using the GST–TANK as substrate was carried out. Anti-TRAF-2 and -NEMO Western blots performed on the cell lysates are illustrated (second and third panels from the top respectively). (**D**) IKK-mediated phosphorylation of TANK requires RIP1. Wild-type or RIP1-deficient Jurkat cells were left untreated or stimulated with TNFα for the indicated periods of time and *in vitro* kinase assays using the GST–TANK as substrate were carried out. Anti-RIP1 and -NEMO Western-blot analyses were performed with the cell extracts (second and third panels from the top respectively). IP, immunoprecipitation.

forms disappeared in BAY 11-7085-pretreated cells subjected to TNFα stimulation (results not shown).

The TNFα-mediated signalling pathway leading to NF-κB activation requires RIP1 and TRAF-2 and is triggered upon binding of this cytokine to two distinct receptors, namely the TNFR1 and the TNFR2. Therefore we next investigated whether these essential proteins are required for TNFα-mediated TANK phosphorylation in cells specifically deficient for each of them by kinase assays (Figure 4). First, TNFα-mediated TANK phosphorylation was observed in TNFR2 but not in TNFR1 KO cells (Figures 4B and 4A respectively, upper panels), suggesting that only the TNFR1 is required. Of note, we noticed a delayed TANK phosphorylation in TNFR2 KO cells compared with wild-type cells

(Figure 4B, compare upper panels). Because TRAF-2 is an adaptor protein connecting the TNFR1 signalling complex to the IKKs [4], we next investigated whether TANK is phosphorylated in TRAF-2-deficient MEF cells (Figure 4C). A TNFα-dependent TANK phosphorylation was detected in TRAF-2-deficient MEFs, although it was sustained when compared with wild-type cells (Figure 3C, compare top panels). This may indicate that TRAF-2 negatively regulates TNFα-mediated TANK phosphorylation. Because TRAF-5 may compensate for TRAF-2 function in TRAF-2 KO cells, as evidenced by the lack of TNFα-mediated NF-κB activation in TRAF-2 and -5 KO cells [31], the use of these cells may clarify this issue.

The kinase RIP1 is also recruited to the TNFR1 in response to TNFα [32]. In order to define its role in IKKβ-mediated TANK phosphorylation, wild-type as well as RIP1-deficient Jurkat cells were stimulated with TNFα and kinase assays using the GST–TANK as substrate were carried out. Here again, the TNFα-dependent TANK phosphorylation was impaired in RIP1-deficient Jurkat cells (Figure 4D, top panels). Therefore TNFα-mediated TANK phosphorylation requires RIP1.

IKK-mediated phosphorylation of TANK occurs on the C-terminal end of the scaffold protein

The experiments described above strongly suggest that TANK is phosphorylated by IKKβ within its C-terminal domain (see Figure 2D). Therefore, in an attempt to more precisely identify the TANK residues targeted for phosphorylation by the IKK complex, we first compared the amino acid sequence of this protein with the other known substrates of the IKKs such as IκBα [33] and IRS-1/2 (insulin receptor substrate 1/2) [34] and identified a potential site within the C-terminal domain of TANK (Figure 5A). Indeed, the 'DSVVLS' sequence from amino acids 379–384 of TANK matched the consensus sequence of the IKK phosphorylating sites ('DSXXXS'). Importantly, other potential phosphorylated residues are within the C-terminal domain of TANK as well. Therefore we first mapped the targeted TANK domain by incubating anti-NEMO or -HA (negative control) immunoprecipitates from TNFα-treated or unstimulated HEK-293 cells with various GST–TANK fusion products harbouring distinct domains of this scaffold protein and named 'GST 2 to 4' (Figure 5B). As expected, a TNFα-dependent phosphorylation was detected using 'GST 2' (amino acids 282–425) as substrate (Figure 5B, upper panel, lanes 3 and 4). Therefore the last 143 amino acids of TANK are targeted by the IKK complex upon TNFα stimulation. Because the 'DSVVLS' sequence is located within this domain, we repeated this experiment using the GST–TANK fusion protein named 'GST 2 4*' where Ser^{378}, Ser^{380}, Ser^{384} and Ser^{388} were all mutated to alanines. Unexpectedly, this substrate was still phosphorylated upon TNFα stimulation (Figure 5B, upper panel, lanes 7 and 8), suggesting that TANK residues other than the 'DSXXXS' consensus sequence and within the last 143 amino acids are targeted by the IKK complex. An additional GST–TANK fusion protein harbouring amino acids 282–374 and named 'GST 3' was used as substrate in a subsequent kinase assay. A very weak phosphorylation of this product was detectable upon TNFα stimulation, whereas a GST–TANK fusion protein harbouring the last 51 C-terminal residues (amino acids 375–425) and named 'GST 4' was more intensively phosphorylated upon TNFα stimulation (Figure 5B, upper panel, lanes 9–12, 15 and 16). Among these last 51 C-terminal residues, six were potential phosphorylation sites. They were individually mutated and the mutants were tested for phosphorylation but none of them showed a significantly reduced phosphorylation (results not shown). Therefore IKKβ-mediated TANK phosphorylation occurs on multiple sites within this domain, similarly to what has been seen for IKKε-mediated TANK phosphorylation (A. Chariot and U. Siebenlist, unpublished work). Taken together, these results suggest that the last 51 amino acids domain of TANK harbour the residues targeted for phosphorylation by IKKβ.

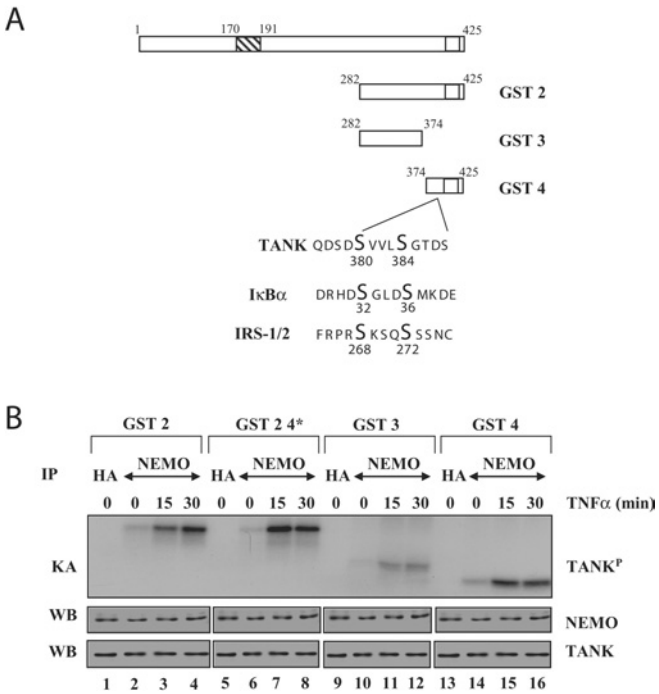

Figure 5 TNFα-mediated phosphorylation of TANK targets the C-terminal domain of this scaffold protein

(**A**) Schematic representation of the TANK protein with the potential C-terminal phosphorylated residues upon TNFα stimulation. (**B**) TANK is phosphorylated within its last 51 amino acids of the C-terminal domain upon TNFα stimulation. HEK-293 cells were left untreated or stimulated with TNFα for the indicated periods of time. Cell extracts were subjected to anti-HA (negative control) or anti-NEMO immunoprecipitations (IP) followed by kinase assays (KA) using these immunoprecipitates and a GST–TANK fusion protein as substrate. WB, Western blot.

TNFα-mediated TANK phosphorylation negatively regulates its ability to associate with NEMO

To explore the consequences of TANK phosphorylation on its activity, we determined whether or not this post-translational modification regulates TANK's ability to associate with known partners such as NEMO. Only the unphosphorylated form of TANK was associated with NEMO (Figure 6, left panel, lanes 2 and 3), even when phosphorylated forms of TANK were detectable in the cell lysates because of IKKβ overexpression (Figure 6, top right panel, lane 3). Moreover, IKKβ overexpression actually attenuated the ability of TANK to interact with NEMO (Figure 6, left panel, compare lanes 2 and 3). Therefore IKKβ-mediated TANK phosphorylation prevents its association with NEMO.

TANK is required for TNFα-mediated NF-κB activation by regulating p65 transactivation potential

Because TANK recruitment to the IKK complex is enhanced by TNFα stimulation, we next explored whether and how this scaffold protein plays a role in the TNFα-mediated NF-κB activation signalling pathway. To do so, we determined whether

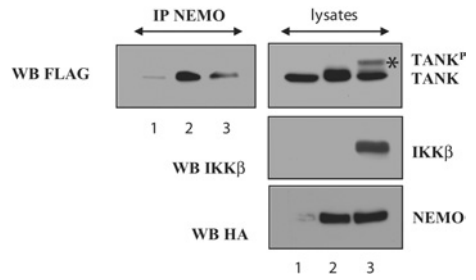

Figure 6 IKKβ-mediated TANK phosphorylation attenuates its interaction with NEMO

HEK-293 cells were transfected with FLAG–TANK alone (lane 1) or with HA–NEMO only or in combination with HA–IKKβ (lanes 2 and 3 respectively) and anti-NEMO immunoprecipitations (IP) were performed on the cell extracts followed by anti-FLAG Western-blot (WB) analyses (left panel). Anti-FLAG, -IKKβ and -HA Western-blot analyses were also carried out on cell lysates (right panels). The asterisk illustrates the phosphorylated form of TANK (TANK^P).

decreased TANK expression through RNAi impaired this signalling pathway. HEK-293 cells were transfected with an siRNA (small interfering RNA) targeting either the TANK transcript or GFP (green fluorescent protein) as negative control. The next day, cells were transfected with a κB site-containing reporter plasmid and were left unstimulated or treated with TNFα and luciferase assays were performed. Cells transfected with the GFP siRNA harboured normal TNFα-mediated NF-κB activation, whereas this activation was altered in cells transfected with TANK siRNA (Figure 7A), suggesting that TANK is required for an optimal NF-κB activation in response to this cytokine. Therefore this genetic approach clearly proves for the first time that TANK positively regulates NF-κB in this pathway.

In order to provide additional experimental evidence for this model, we next tested the ability of TANK to activate NF-κB when co-expressed or not with one of its interacting partner, namely NEMO, by luciferase assays. Neither TANK nor the tested TANK mutants (TANKΔIKKε which lacks the IKKε/TBK1-interacting site, as well as TANKΔN70 and ΔC178 which lack the N- and C-terminal NEMO-interacting sites respectively) modulated NF-κB activation when expressed alone (Figure 7B). However and importantly, TANK activated NF-κB when co-expressed with NEMO and this activation was impaired when NEMO was expressed with the tested TANK mutants. Therefore this result reinforces the hypothesis that TANK positively regulates NF-κB activation and also demonstrates the critical role of the IKKε/TBK1- and NEMO-interacting domains of TANK in this pathway.

TNFα-mediated NF-κB activation requires IKK-dependent IκBα phosphorylation and subsequent degradation [2] and also a phospho-dependent p65 enhanced transactivation potential [15]. Therefore we explored through which mechanism(s) TANK regulates this signalling pathway by investigating whether TNFα-mediated IKK activation requires TANK. HEK-293 cells transfected with either TANK or GFP siRNAs were left unstimulated or treated with TNFα for the indicated periods of time and IKK activation was assessed by *in vitro* kinase assays. The profile of TNFα-mediated IKK activation was similar in GFP and TANK RNAi cells (Figure 7C, upper panel), suggesting that IKK activation in response to this cytokine does not require TANK.

To investigate whether TANK is involved in TNFα-mediated up-regulation of the p65 transactivation potential, GFP or TANK RNAi HEK-293 cells were transfected with a reporter plasmid harbouring GAL4 DNA binding responsive elements with or without a p65 construct encompassing the C-terminal transactivation domain fused to the GAL4 DNA-binding domain ['GAL4-p65 (286–551)']. Cells were subsequently left unstimulated or treated with TNFα and the p65 transactivation potential was assessed by measuring luciferase activities. Whereas TNFα enhanced p65 transactivation potential, decreased TANK expression severely impaired both basal and signal-induced p65 transactivation abilities (Figure 7D). Therefore our results indicate that TANK is required for the basal NF-κB activity and also positively regulates p65 transactivation potential, probably by

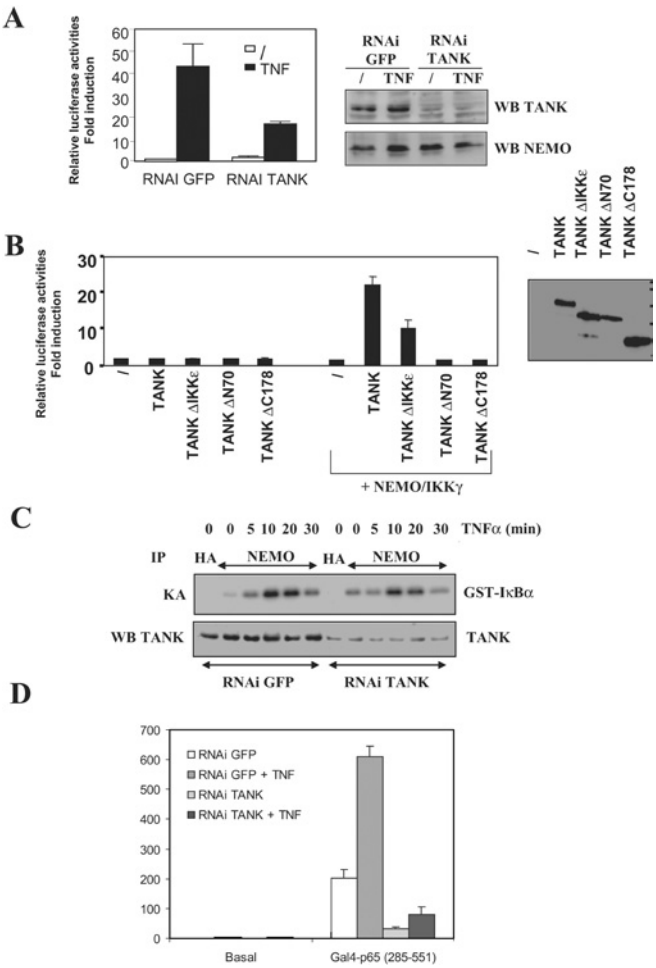

Figure 7 TANK positively regulates NF-κB activation in response to TNFα

(**A**) Decreased TNFα- and NF-κB-mediated gene activation in TANK RNAi cells. HEK-293 cells were transfected with 0.2 μg of RSV-βgal (where RSV is Rous sarcoma virus) and 1 μg of the κB luciferase reporter and were either left untreated or stimulated with TNFα (100 units/ml) for 5 h before lysis, as indicated. The cell extracts were collected for the measurement of both luciferase and β-galactosidase activities. The results of three independent experiments performed in triplicate, after normalization with β-Gal activities, are shown (means ± S.D.). Anti-TANK and -NEMO Western blots (WB) performed on representative extracts are shown. RNAi, RNAi. (**B**) Wild-type TANK but not TANK mutants strongly activates NF-κB when co-expressed with NEMO. HEK-293 cells were transfected with the κB luciferase reporter, the RSV-βgal as well as the indicated expression vectors. Cell extracts were collected for the measurement of both luciferase and β-galactosidase activities and the results are shown as described above. An anti-FLAG Western blot performed to assess the levels of expression for each FLAG-tagged TANK expression vector is also illustrated. (**C**) IKK activation is unaltered in TANK RNAi cells. GFP or TANK RNAi cells were left unstimulated or treated with TNFα for the indicated periods of time and anti-NEMO immunoprecipitates (IP) were subjected to *in vitro* kinase assays (KA) using a purified GST–IκBα protein as substrate. Anti-TANK Western-blot analyses were also carried out on cell extracts. (**D**) TANK positively regulates p65 transactivation potential. Cells were transfected with a reporter plasmid harbouring a GAL4 DNA-responsive element (see the Materials and methods section) either alone ('basal') or in combination with the indicated GAL4–p65 fusion protein. p65 transactivation potential was assessed by measuring luciferase activities. The results of one representative experiment performed in triplicate, after normalization with *Renilla* luciferase activities, are shown (means ± S.D.).

connecting upstream signalling molecules to the IKK complex and this NF-κB protein.

Apart from TRAF-2, TANK has previously also been shown to bind to IKKε and TBK1 [20,21]. To investigate whether TNFα modulates the interaction of TANK with any of these kinases, anti-TANK immunoprecipitations were performed with cell extracts from HEK-293 cells either left untreated or stimulated with TNFα. As expected, IKKε and TBK1 were found in such immunoprecipitates and their interaction with TANK was not modulated upon treatment with TNFα (Supplementary Figure S1, http://www.BiochemJ.org/bj/394/bj3940593add.htm). To identify novel TANK-interacting kinases that might be involved in the TANK-mediated NF-κB activation pathway triggered by TNFα, we tested a number of candidates besides TBK1/T2K [35] whose loss-of-function experiments indeed showed impaired TNFα-mediated NF-κB activation, namely TAK1 [8–10], MEKK3 [11] and Tpl2/Cot [36], but also kinases known to phosphorylate p65 in response to TNFα such as CK2 [37], but none of these kinases associated with TANK (Supplementary Figure S1). Taken together, our results demonstrate that TBK1 is the only TANK-interacting kinase whose activity has been previously linked with TNFα-mediated NF-κB activation [35], suggesting a potential role of this kinase in the regulation of TNFα-induced NF-κB-dependent gene expression through TANK.

TANK is required for TNFα-mediated expression of a subset of target genes

To identify the target genes whose TNFα-mediated induction requires TANK, we first identified the ones whose expression is induced by TNFα in HeLa cells by micro-array analyses. As expected, numerous genes coding for cytokines were strongly induced by TNFα and included those encoding IL-8 (interleukin-8), IL-6 and IL-1α, as well as TNFα (results not shown). We next transfected TANK and GFP siRNAs in HeLa cells, left them unstimulated or treated them with TNFα and investigated the expression of selected target genes by real-time PCR. As expected, TANK RNA level was decreased in cells transfected with the TANK siRNAs (Figure 8). Interestingly, whereas TNFα-mediated IL-8 induction was confirmed in GFP RNAi cells, induction of this target gene was impaired in TNFα-stimulated TANK RNAi HeLa cells (Figure 8). On the other hand, the TNFα-mediated induction of *Gro-beta* (growth-regulated oncogene-beta) expression was not altered by decreased TANK expression. Therefore our results indicate that TANK is required for the expression of some but not all TNFα-mediated NF-κB target genes.

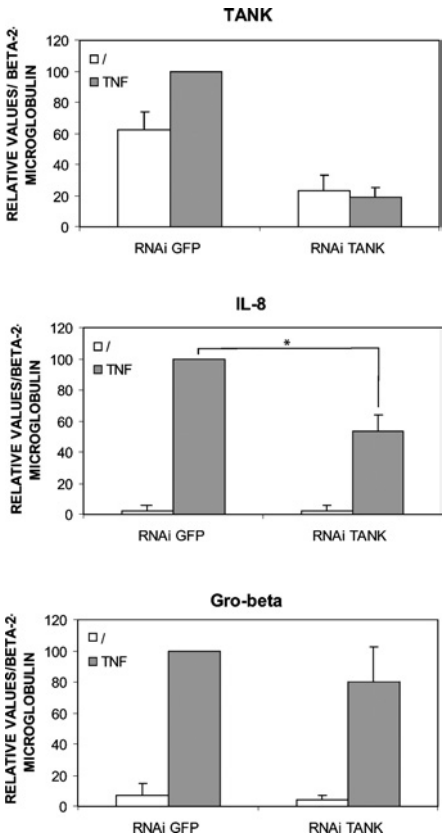

Figure 8 Identification of selected TNFα-induced genes that require TANK

Total RNAs from GFP or TANK RNAi HeLa cells either untreated or stimulated with TNFα were subjected to reverse transcription followed by real-time PCR analyses using the appropriate primers for amplification of the TANK, IL-8 or Gro-beta cDNAs. Amplification of the β_2-microglobulin cDNA was used for normalization purposes. The results of four independent experiments performed in duplicate are shown (means $\pm$ S.D.) and analysed by the Mann–Whitney test ($^*P < 0.05$).

DISCUSSION

A role for the TRAF-binding protein TANK in NF-κB-dependent signalling pathways was reported several years ago but the underlying mechanism remained unknown [19,22,23]. We subsequently demonstrated that TANK associates with the essential protein NEMO, but the physiological signal that triggers this interaction was not identified [24]. We demonstrate here that the scaffold protein TANK is recruited to the IKK complex upon TNFα stimulation and is required for NF-κB activation in response to this pro-inflammatory cytokine, as shown by results obtained with our cellular model of loss-of-function for TANK. Interestingly, TANK deficiency through RNAi does not affect TNFα-mediated IKK activation but rather impairs TNFα- and NF-κB-mediated gene activation through decreased p65 transcriptional potential. It is, however, important to highlight that residual levels of TANK were still detectable in the TANK RNAi cells. Therefore minimal amounts of TANK may be sufficient for TNFα-mediated IKK activation, similarly to what has been observed for NEMO (M. Bonif and A. Chariot, unpublished work).

In any case, our results demonstrate that TANK is involved in the regulation of p65 activity, presumably by connecting an upstream kinase to the IKK complex and p65. One candidate is TBK1, a kinase isolated as a TANK-binding protein [21] and which has been shown to enhance TANK binding to NEMO in transfected cells [24]. This model would imply a role for TBK1 in TNFα-mediated NF-κB activation. Although it has been recently demonstrated that TBK1 is recruited to the TNFR1 upon TNFα stimulation [38], a direct role of TBK1 in this pathway remains controversial. Indeed, initial reports strongly supported a role for TBK1/T2K in NF-κB activation as mice deficient for *tbk1/t2k* died of massive liver apoptosis *in utero* [35], similarly to the mice deficient for the genes encoding p65, IKKβ or NEMO [39–43]. Interestingly and in contrast with these latter KO phenotypes, T2K-deficient embryonic fibroblasts showed no defect in cytokine-induced liberation of NF-κB from their IκB inhibitors [35]. Instead, impaired NF-κB activation in these cells was rather due to a NF-κB-directed transcription defect since the expression of some but not all target genes such as ICAM-1 and TLR-2 was altered in response to TNFα. It is now established that TBK1 deficiency does not alter p65 translocation and subsequent NF-κB DNA-binding activity in response to TNFα, IL-1β

or lipopolysaccharide [44]. Based on these observations, it is believed that TBK1/T2K may rather target NF-κB transactivation functions and this hypothesis was later experimentally validated by demonstrating that TBK1 phosphorylates p65 on Ser^{536} [45,46] when associated with the scaffold protein NAP1 (NAK-associated protein 1) [45]. Moreover, the observation that deficiency for TANK or for its interacting kinase TBK1/T2K [35] has similar consequences with respect to IKK activation and p65 activity reinforces the hypothesis that both proteins are part of the same functional complex. Another report, however, challenged the role of TBK1 in TNFα-mediated NF-κB activation [47]. Indeed, a recent re-examination of TBK1 KO cells did not show any defect of NF-κB activation induced by viral infection, TLR (Toll-like receptor) ligands or TNFα using three distinct NF-κB reporters [47]. Therefore the role of TBK1 in NF-κB activation may be cell-type-specific, as demonstrated for other kinases such as Tpl2/Cot [36] and PKCζ (protein kinase Cζ) [48].

IKKε may be another p65 kinase because this protein interacts with TANK, but a role in TNFα-mediated NF-κB activation is unlikely since this kinase is not activated by this cytokine, even if TNFα induces IKKε expression [49]. Another still unidentified kinase distinct from the IKKs, TBK1 or IKKε is responsible for p65 phosphorylation on Ser^{536} and subsequent IL-8 expression, as recently shown [46]. Irrespective of which kinase(s) is involved in p65 phosphorylation, and because cells deficient for TANK have impaired TNFα-mediated IL-8 expression, it is tempting to speculate that this kinase activity may be connected to the IKK complex via TANK. Because p65 transactivation potential is modulated through TNFα-mediated phosphorylation by multiple kinases [45], future studies will be dedicated to the identification of the p65 residues that are phosphorylated via the TANK-dependent pathway(s).

Although the present paper strongly suggests a positive role for TANK in TNFα-mediated NF-κB activation, previous over-expression studies with TANK and/or TANK mutants suggested a dual role for this protein [19,22,23]. Indeed, the TANK N-terminal domain which physically interacts with IKKε and TBK1 was shown to exert a positive effect on NF-κB activation, whereas an inhibitory function was attributed to its C-terminal domain, presumably by sequestering TRAF-2 from the signalosome. Our loss-of-function experiments favour the idea that TANK positively regulates NF-κB activation but this does not rule out the possibility that TANK may first activate NF-κB and subsequently inhibits this pathway through as yet unidentified mechanisms that may depend on TANK levels of expression in the cell.

Previous reports demonstrated that TANK is phosphorylated by IKKε, and TBK1 *in vitro*, but the physiological relevance of these observations remains unclear [20,24]. We show here that TANK is phosphorylated by IKKβ through a TNFR1- and RIP1-dependent pathway upon TNFα stimulation *in vitro* and *in vivo*. We noticed a delayed TANK phosphorylation in TNFR2-deficient cells which may fit with the described role of TNFR2 in accelerating TNFR1 signals in several cell types [50–52]. The underlying mechanism was suggested to involve TNFR2-mediated TRAF-2 degradation. In the context of TNFα-mediated TANK phosphorylation, this would mean that TRAF-2 somehow inhibits TANK phosphorylation and this hypothesis is actually supported by the sustained TANK phosphorylation in TRAF-2-deficient cells (see Figure 4C). Of note, the timing of this TANK phosphorylation differs in *in vitro* kinase assays and in *in vivo* phospholabelling experiments and the relevance of this discrepancy, if any, is currently unclear. In any case, we show that this post-translational modification negatively regulates TANK's ability to interact with NEMO. This result combined with, first, the optimal recruitment of TANK to the IKK complex after 15 min (Figure 1A), and secondly, the TANK phosphorylation after 30 min of stimulation *in vivo* (Figure 2E), prompted us to suggest that this phosphorylation may be a mechanism to end the TANK-dependent wave of NF-κB activation by releasing this scaffold protein from the IKK complex.

Our results reveal that decreased TANK expression has distinct consequences for the expression of selected target genes in response to TNFα. Indeed, whereas TNFα-mediated *Gro-beta* induction was not impaired in TANK RNAi cells, IL-8 induction was altered in such cells. In this context, recent studies showed that p65 phosphorylation on specific residues is crucial for the expression of a subset of NF-κB target genes. For example, p65 phosphorylation on Ser^{276} is required for TNFα-mediated IL-6 expression [53], whereas p65 phosphorylation on Ser^{536} helps the binding of this NF-κB protein to the promoter of IL-8 in response to IL-1β [46]. When combined with our results, these observations also suggest that the TANK-dependent signalling pathways triggered by TNFα stimulation target specific p65 residues for phosphorylation and control of a subset of TNFα-induced genes. Such a hypothesis does not rule out the possibility that other post-translational modifications such as p65 acetylation may be impaired in TANK-deficient cells as well.

It is important to remember that TNFα induces gene expression also through NF-κB-independent signalling pathways such as the ones leading to SAPK (stress-activated protein kinase)/c-Jun N-terminal kinase activation. In this context, the stress-responsive p38 MAPK is indeed activated upon TNFα stimulation and this MAPK-dependent pathway is required for subsequent IL-6 expression [54]. In addition and because TANK was also found associated with GCKR (germinal centre kinase-related kinase) [29], another kinase known to play a role in the SAPK-dependent pathways triggered by TNFα stimulation, it is likely that the defects in TNFα-mediated target genes in the TANK RNAi cells may also be the result of impaired NF-κB-independent signalling pathways. Interestingly, the TNFα-and NF-κB-dependent target genes have been identified in epidermal keratinocytes [55]. These NF-κB-dependent genes code for proteins involved in cell motility, cytoskeletal changes, inflammation, immune response and apoptosis, whereas the genes coding for cell cycle proteins or metabolic pathways are induced through NF-κB-independent pathways. In these cells, IL-8 expression is NF-κB-dependent [55], which reinforces our hypothesis that TANK plays a role in the TNFα- and NF-κB-mediated signalling pathways. An extensive study of all the TNFα-mediated target genes in GFP versus TANK RNAi cells will undoubtedly help us to decipher the roles played by TANK in all the signalling pathways triggered by this cytokine.

The scaffold protein TANK may exist in distinct cytoplasmic pools, similarly to the TRAF proteins. Indeed, TANK is a TRAF-2-interacting molecule and consequently takes part in the signalling pathway triggered by TNFα stimulation as demonstrated here. On the other hand, TANK is constitutively associated with the kinases IKKε and TBK1, which are required for phosphorylation of the IRF-3 (interferon regulatory factor-3) protein in response to viral infection via the TLR3 [56,57]. Although the potential role of TANK in this latter signalling pathway remains to be demonstrated, this suggests that TANK and its interacting kinases play critical roles in other, NF-κB-independent, pathways, such as the ones leading to IRF activation.

In conclusion, we suggest a model where TNFα binding to the TNFR1 triggers the recruitment of the IKK complex to the cell membrane, which implies an association of NEMO with TANK. This latter interaction connects TBK1 to the IKK complex which will subsequently enhance p65 transactivation potential. Meanwhile, IKKβ-mediated TANK phosphorylation attenuates

its binding to the IKK complex and may therefore terminate the TANK-dependent wave of NF-κB activation.

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