The EMBO Journal Vol.18 No.23 pp.6694–6704, 1999

NF-κB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase

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The activation of NF-κB by receptors in the tumor necrosis factor (TNF) receptor and Toll/interleukin-1 (IL-1) receptor families requires the TRAF family of adaptor proteins. Receptor oligomerization causes the recruitment of TRAFs to the receptor complex, followed by the activation of a kinase cascade that results in the phosphorylation of IκB. TANK is a TRAF-binding protein that can inhibit the binding of TRAFs to receptor tails and can also inhibit NF-κB activation by these receptors. However, TANK also displays the ability to stimulate TRAF-mediated NF-κB activation. In this report, we investigate the mechanism of the stimulatory activity of TANK. We find that TANK interacts with TBK1 (TANK-binding kinase 1), a novel IKK-related kinase that can activate NF-κB in a kinase-dependent manner. TBK1, TANK and TRAF2 can form a ternary complex, and complex formation appears to be required for TBK1 activity. Kinase-inactive TBK1 inhibits TANK-mediated NF-κB activation but does not block the activation mediated by TNF-α, IL-1 or CD40. The TBK1–TANK–TRAF2 signaling complex functions upstream of NIK and the IKK complex and represents an alternative to the receptor signaling complex for TRAF-mediated activation of NF-κB.
Keywords: IKK/NF-κB/TANK/TBK1/TRAF2

Introduction

NF-κB is a ubiquitously expressed transcription factor that regulates the induction of genes involved in immune and inflammatory cell function (Verma *et al*., 1995; Baldwin, 1996), and in anti-apoptotic responses (Beg and Baltimore, 1996; Liu *et al*., 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996). NF-κB is composed of homo- and heterodimers of Rel family members, typically p65:p50, which are held in the cytoplasm by the inhibitory IκB proteins (IκBα, IκBβ or IκBε). NF-κB activation results from the phosphorylation of IκB on two conserved serine residues near its N-terminus, which targets it for ubiquitylation and degradation by the 26S proteasome. After the destruction of IκB, NF-κB can then translocate to the nucleus to activate target genes.

The signal-induced phosphorylation of IκB is carried out by an IκB–kinase complex (IKK complex) that contains two kinase subunits, IKKα and IKKβ, as well as one or more scaffolding proteins including NEMO and IKAP (Zandi and Karin, 1999). The IKK complex receives signals from the tumor necrosis factor (TNF) and Toll/interleukin-1 (IL-1) receptor families, from CD3/CD28 co-stimulation of T cells and from the LMP-1 signaling protein of Epstein–Barr virus (EBV), and is also the target of the *Tax* gene product of HTLV-1 (Zandi and Karin, 1999).

The IKK complex is activated by the NIK kinase (Malinin *et al*., 1997), and other MAP3K kinases including MEKK1, 2 and 3 (Lee *et al*., 1998; Nakano *et al*., 1998; Nemoto *et al*., 1998; Zhao and Lee, 1999). NIK is a potent activator of NF-κB, and kinase-dead NIK blocks NF-κB activation by TNF-α, IL-1, Fas (Malinin *et al*., 1997), Toll-like receptors 2 and 4 (Kirschning *et al*., 1998; Muzio *et al*., 1998), LMP-1 (Sylla *et al*., 1998) and CD3/CD28 stimulation (Lin *et al*., 1999). NIK binds to and activates both IKKα and IKKβ (Regnier *et al*., 1997; Woronicz *et al*., 1997; Ling *et al*., 1998), and NIK itself is activated through phosphorylation in its activation loop, at Thr559 (Lin *et al*., 1998).

Receptor oligomerization is thought to be linked to the activation of the NIK–IKK kinase cascade by members of the TRAF family of adaptor proteins (Arch *et al*., 1998). It appears that TRAFs are recruited to ligand-bound receptors either through direct binding to cytoplasmic signaling domains (e.g. CD40, CD30, CD27, LMP-1 and TNFR2) or by binding to other adaptor proteins that bind directly to receptor tails (e.g. TRADD and RIP for TNF receptor 1, MyD88 and IRAK for the IL-1 receptor). TRAFs 2, 5 and 6 have been shown to activate NF-κB and JNK1 when overexpressed in tissue culture cells (Rothe *et al*., 1995; Cao *et al*., 1996; Nakano *et al*., 1996; Song *et al*., 1997). All three interact directly with NIK (Song *et al*., 1997), but it is unclear what precise mechanistic role TRAFs play in NIK activation.

TANK (I-TRAF) is a TRAF-binding protein with both stimulatory and inhibitory properties (Cheng and Baltimore, 1996; Rothe *et al*., 1996). The stimulatory activity of TANK is manifested most clearly in the ability to activate NF-κB synergistically with low levels of TRAF2 when expressed in tissue culture cells (Cheng and Baltimore, 1996). This activity requires the N-terminal domain (residues 1–168) of TANK as well as a central region (169–190) that is required for interaction with TRAF2 (Cheng and Baltimore, 1996). The C-terminal domain (190–413) of TANK has been shown to inhibit the activity of the N-terminal domain *in trans*, as well as to inhibit activation of NF-κB by CD40 (Cheng and Baltimore, 1996). These observations implicated a role for TANK in TRAF2-dependent pathways that activate NF-κB such as those downstream of CD40L and TNF-α. However, other experiments revealed that TANK could inhibit the activation of NF-κB by TNF-α, IL-1, CD40, LMP-1 and high levels of transfected TRAF2 (Kaye *et al*., 1996; Rothe *et al*., 1996). This TANK-mediated inhibition

was attributable to the inhibitory activity of the C-terminal domain, and also to the ability of the TRAF-interacting region of TANK to compete directly for TRAF2 binding to signaling domains of CD40, TNFR2 and LMP-1 (Cheng and Baltimore, 1996; Kaye *et al*., 1996; Rothe *et al*., 1996). In the absence of a TANK-deficient cell line or knowledge of TANK's mechanism of action, it has remained unclear whether TANK plays an obligate role in NF-κB activation by these cell surface receptors.

The mechanism by which TANK modulates TRAF2 function has not been well defined. For example, it was not known whether the synergy observed between TANK and TRAF2 reflected action by each protein on the same downstream cascade of molecules, namely NIK and the IKK complex, or whether TANK affected an undefined parallel pathway that also leads to IκB phosphorylation. Furthermore, it was unknown whether TANK could serve as a cofactor for other TRAF family members that also activate NF-κB, such as TRAF5 and TRAF6. While TANK modulated TRAF2-mediated NF-κB activation, it has not been addressed whether TANK affected JNK1 activation by TRAF2. Because of the lack of homology of TANK to any known protein, its mechanism of action was not predictable.

In this report we describe the identification and characterization of a novel kinase, TBK1, which mediates TANK's ability to activate NF-κB. TBK1 functions in a TBK1–TANK–TRAF2 signaling complex that feeds into the NIK–IKK cascade, but is not required for NF-κB activation by TNF-α, IL-1 or CD40. TANK emerges as a bifunctional adaptor protein, functioning as an inhibitor of TBK1-independent pathways and as a cofactor for stimulation in a TBK1-dependent pathway.

Results

To investigate the mechanism by which TANK stimulates NF-κB, we constructed TANK 1–190 because it includes the stimulatory domain (residues 1–168) and the region required for TRAF2 interaction (169–190), and it lacks the inhibitory domain (residues 190–413). We compared TANK 1–190 with full-length TANK using a κB-dependent reporter in transient transfections in 293 cells (Figure 1). As shown previously (Cheng and Baltimore, 1996), full-length TANK displayed minimal stimulatory activity in the absence of co-transfected TRAF2, and a biphasic profile of stimulation in the presence of a low level of co-transfected TRAF2 (Figure 1A). TANK 1–190 elicited a much more robust response, stimulating up to 17.5-fold in the absence of co-transfected TRAF2, and synergizing with TRAF2 to a much greater extent than full-length TANK (Figure 1B). In addition, while full-length TANK was inhibitory when co-expressed with higher concentrations of TRAF2 (Figure 1C), TANK 1–190 was slightly stimulatory. TANK 1–190 thus afforded a tool to assay TANK stimulatory activity in a robust manner in the absence of TANK inhibitory activity.

Dominant-negative forms of TRAF2, NIK, IKKα and IKKβ were each able to inhibit the stimulation of the κB-dependent reporter by TANK 1–190 (Figure 2). These data suggest that TANK potentiates the TRAF2-mediated activation of NIK and the subsequent activation of the IKK complex.

Since members of the TRAF family have distinct functional specificities (Arch *et al*., 1998), we tested whether TANK might also stimulate the κB-dependent reporter in conjunction with TRAF5 and TRAF6, two other TRAFs that also stimulate NF-κB (Figure 3). TANK 1–190 synergistically activated NF-κB with both TRAF5 and TRAF6, and is therefore a general coactivator.

Isolation of TBK1 in two-hybrid screening with TANK

The lack of homology in the stimulatory region of TANK to any known signaling domain offered no insight into the mechanism of TANK function. To identify other proteins that might function with TANK, a two-hybrid screen was performed using TANK 1–190 fused to GAL4 as bait, and a human B-cell cDNA library fused to the GAL4 activation domain. Of 7.4×10^5 transformants screened, 23 were positive and confirmed to be lacZ positive. Of these, eight represented overlapping cDNAs of a single gene. We have named this gene TBK1 (TNK-binding kinase 1). The amino acid sequences of human and murine TBK1 are 94% identical and are shown in Figure 4A. Murine TBK1 was studied in all subsequent experiments.

TBK1 is a 729 amino acid protein containing a kinase domain at its N-terminus. Two putative coiled-coil-containing regions are found within its C-terminal region (residues 603–650 and 679–712). The known kinases that are most homologous to TBK1 are IKKα and IKKβ, which are each 27% identical and 45% similar in the region between TBK1 residues 9 and 353 (Figure 4B). Northern blot analysis revealed a single ~3.3 kb transcript expressed in mouse stomach, small intestine, lung, testis, skin, brain, heart, kidney, spleen, thymus and liver (data not shown).

The smallest cDNA activation domain fusion isolated in two-hybrid analysis contained only the C-terminal 43 residues of TBK1 (Figure 4A), indicating that this region is sufficient for interaction with TANK.

TBK1 activates NF-κB

If TBK1 mediates the ability of TANK to activate NF-κB, one might expect it to induce κB DNA-binding activity and activate the κB-dependent reporter when overexpressed. Indeed, TBK1 induced p50:p65 DNA-binding activity in 293 cell nuclear extracts (Figure 5A) and stimulated the κB reporter in a dose-dependent manner, stimulating up to 260-fold at the highest concentration tested (Figure 5B, squares). These activities were dependent on an intact kinase domain because mutation of Lys38 (a lysine conserved in kinases and necessary for catalytic function) to alanine completely abrogated the stimulatory activity of TBK1 (Figure 5A and B, diamonds). TBK1 did not activate a control reporter that did not contain NF-κB-binding sites (data not shown).

TBK1 was co-transfected with the same dominant-negatives that inhibited TANK activity. As shown in Figure 5C, the RING finger-deleted TRAF2 construct inhibited TBK1 activity, as did kinase-dead versions of NIK, IKKα and IKKβ. We also observed that an activation-incompetent NIK (T559A in the activation loop) could

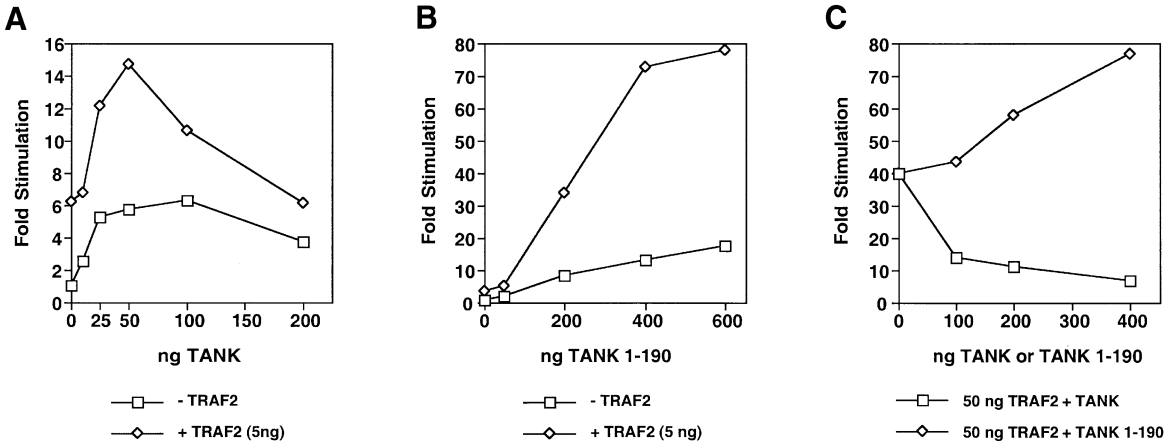

Fig. 1. Comparison of TANK and TANK 1–190 in the absence and presence of large and small amounts of TRAF2. (**A**) The TANK expression vector was titrated into transfections in 293 cells in the absence (□) and presence (◇) of 5 ng of the TRAF2 expression vector and 100 ng of the $(\kappa B)_3$-IFN-LUC reporter; fold stimulation was determined as described in Materials and methods. (**B**) The TANK 1–190 expression vector was titrated as in (A). (**C**) Expression vectors for TANK (□) or TANK1–190 (◇) were titrated in the presence of 50 ng of the TRAF2 expression vector and 100 ng of the $(\kappa B)_3$-IFN-LUC reporter. Western blotting of transfected and untransfected extracts allowed us to estimate that 5 ng of TRAF2 expression vector led to ~2- to 3-fold overexpression relative to endogenous levels of TRAF2, while 50 ng led to ~25-fold overexpression; 25 ng of TANK expression vector led to ~5-fold overexpression, while 100 ng led to ~25-fold overexpression; 200 ng of TANK 1–190 expression vector led to ~3-fold higher expression than the level of endogenous full-length TANK.

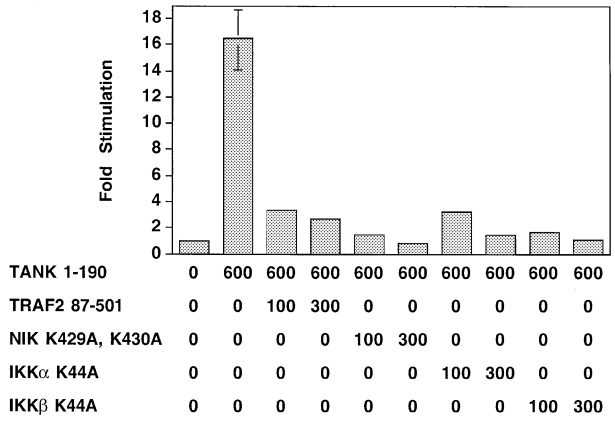

Fig. 2. Dependence of TANK 1–190 on TRAF2, NIK and the IKK complex. The indicated amounts (in ng) of expression constructs for TANK 1–190 and the indicated dominant-negatives were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.00 ± 0.04; 16.5 ± 2.2; 3.3 ± 0.2; 2.7 ± 0.2; 1.5 ± 0.2; 0.8 ± 0.1; 3.2 ± 0.1; 1.5 ± 0.1; 1.6 ± 0.1; and 1.1 ± 0.1.

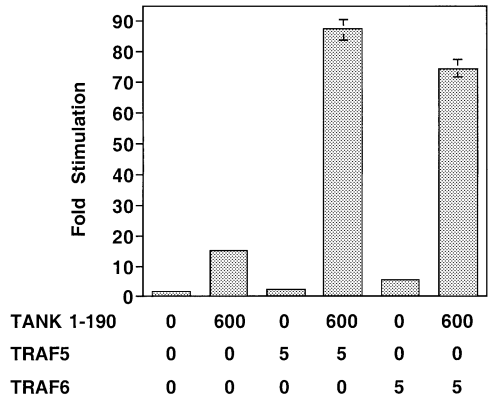

Fig. 3. TANK is a general TRAF cofactor. The indicated amounts (in ng) of expression vectors for TANK1–190, TRAF5 and TRAF6 were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 15.1 ± 1.1; 2.4 ± 0.2; 87.5 ± 3.0; 5.5 ± 0.8; and 74.5 ± 2.9.

block TBK1-mediated activation. These data indicate that TBK1 requires the activities of TRAF2, NIK and the IKK complex, and are consistent with TBK1 mediating the activity of TANK.

TBK1 is required for TANK-mediated NF-κB activation

The TBK1 K38A mutant was tested to see whether it would behave as a dominant-negative for TANK-mediated NF-κB activation. TBK1 K38A inhibited the activation observed with TANK 1–190, as well as the synergistic activation observed with TANK 1–190 and TRAF2 (Figure 6A). These results indicate that TBK1 (or a close relative for which TBK1 K38A is competitive) is required for TANK's ability to activate NF-κB.

Interestingly, TBK1 K38A did not appear to affect the low level of reporter stimulation observed with low levels of TRAF2 alone (Figure 6A), and only mildly reduced the activation observed with higher amounts of TRAF2. TRAF2 activity in this assay appears to be for the most part TBK1-independent in the absence of co-transfected TANK but TBK1-dependent in the presence of co-transfected TANK.

We also tested whether TBK1 K38A would inhibit the reporter stimulation achieved by overexpression of NIK or IKKβ. No effect on NIK- or IKKβ-mediated stimulation was observed (Figure 6B). These results are consistent with the results in Figure 5C indicating that TBK1 functions upstream of TRAF2.

TBK1 K38A also inhibited the stimulation observed with co-expression of TANK 1–190 and either TRAF5 or TRAF6 (Figure 6C), suggesting that TBK1 kinase activity

is required for TANK's ability to serve as a general TRAF cofactor in the synergistic activation of NF-κB.

TBK1 forms a ternary complex with TANK and TRAF2

We tested in co-immunoprecipitation experiments whether TBK1 could interact with TANK in 293 cells and whether TANK could associate with TBK1 and TRAF2 simultaneously. As shown in Figure 7A, hemagglutinin (HA)-tagged TANK 1–190 readily immunoprecipitated with anti-Myc-conjugated agarose only in the presence of Myc-tagged TBK1 (cf. lanes 2 and 3), confirming an association between TANK 1–190 and TBK1. Full-length TANK also readily immunoprecipitated with Myc-TBK1, indicating that the presence of the C-terminal inhibitory domain did not affect TANK–TBK1 association (Figure 7A, lanes 4 and 5). Co-expression of TBK1 and TANK resulted in a slower mobility of TANK on SDS–PAGE that was more apparent with full-length TANK than with TANK 1–190 (Figure 7A, cf. lanes 2–5, lysate). Phosphatase treatment of extracts indicated that this mobility shift was due to phosphorylation (data not shown).

The C-terminal 42 residues of TBK1 were fused to GST and this fusion (GST–TBK1 688–729) was tested for the ability to precipitate full-length TANK and TANK 1–190 with glutathione–Sepharose beads. This portion of TBK1 was sufficient to associate with both full-length TANK (Figure 7A, lanes 10 and 11) and TANK 1–190 (lanes 8 and 9), as suggested by the shortest clone identified in the two-hybrid screen. Co-expression of TANK with GST–TBK1 688–729 did not result in TANK phosphorylation, indicating that while phosphorylated TANK associated with TBK1 (Figure 7A, cf. lanes 3, 5, 9 and 11), phosphorylation of TANK was not required for TBK1 association.

We then tested whether TBK1 could interact with TRAF2 and whether TANK could interact simultaneously with TRAF2 and TBK1. Flag-tagged TRAF2 was immunoprecipitated by anti-Myc-conjugated agarose only in the presence of both HA-TANK and Myc-TBK1, indicating the formation of a TRAF2–TANK–TBK1 ternary complex and arguing against direct association between TBK1 and TRAF2 (Figure 7B, cf. lanes 2, 3 and 4). Both TANK 1–190 and full-length TANK supported the formation of the ternary complex (Figure 7B, lanes 3–6). Interestingly, co-expression of TRAF2 and TBK1 resulted in a modification of TRAF2 revealed by multiple bands of slower mobility (Figure 7B, cf. lanes 1 and 2, lysate). The slower mobility species were present in the ternary complex, because they were immunoprecipitated with TANK and TBK1 (Figure 7B, lanes 4 and 6). Phosphatase treatment of extracts confirmed that this TRAF2 modification was phosphorylation (data not shown). Phosphorylated TRAF2 was present in TBK1–TANK–TRAF2 complexes that contained either TANK 1–190 or full-length TANK (Figure 7B, lanes 4 and 6). The C-terminal 42 residues of TBK1 also precipitated TRAF2 in a TANK-dependent manner (Figure 7B, cf. lanes 7–12), although the expression of GST–TBK1 688–729 did not result in TRAF2 phosphorylation. This result indicates that the kinase domain of TBK1 is not required for this association and that phosphorylation of TRAF2 is not required for formation of the ternary complex.

TBK1 K38A was competent to form the TBK1–TANK–TRAF2 ternary complex, but co-expression of TBK1 K38A did not result in phosphorylation of either TANK or TRAF2 (data not shown), indicating that TBK1 may phosphorylate these proteins directly or activate another kinase which does so.

In other immunoprecipitation experiments, we did not observe evidence for an interaction between TBK1 and either NIK, IKKα or IKKβ (data not shown). In addition, we did not observe an association between NIK and the TBK1–TANK–TRAF2 complex (data not shown).

A

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murine MQSTSNHLWLLSDILGQGATANVFRGRHKKTGDLYAVKVFNNISFLRPVD  50
 human ................................F.I.............

       VQMREFEVLKKLNHKNIVKLFAIEEETTTRHKVLIMEFCPCGSLYTVLEE 100
       ..................................................

       PSNAYGLPESEFLIVLRDVVGGMNHLRENGIVHRDIKPGNIMRVIGEDGQ 150
       ..................................................

       SVYKLTDFGAARELEDDEQFVSLYGTEEYLHPDMYERAVLRKDHQKKYGA 200
       ..................................................

       TVDLWSVGVTFYHAATGSLPFRPFEGPRRNKEVMYKIITGKPSGAISGVQ 250
       ......I...........................................

       KAENGPIDWSGDMPLSCSLSQGLQALLTPVLANILEADQEKCWGFDQFFA 300
       ..............V.....R...V..........................

       ETSDVLHRMVIHVFSLQHMTAHKIYIHSYNTAAVFHELVVKQTKIVSSNQ 350
       ....I............Q..............TI...........I....

       ELIYEGRRLVLELGRLAQHFPKTTEENPIFVTSREQLNTVGLRYEKISLP 400
       ............P..................V...P...I..I.......

       KIHPRYDLDGDASMAKAVTGVVCYACRTASTLLLYQELMRKGVRWLVELV 450
       .V..............I.........I..............I...I..I

       KDDYNETVHKKTEVVITLDFCIRNIEKTVKVYEKLMKVNLEAAELGEISD 500
       ....................................I.............

       IHTKLLRLSSSQGTIESSLQDISSRLSPGGLLADTWAHQEGTHPRDRNVE 550
       ................T.....D.......S...A.........K.....

       KLQVLLNCITEIYYQFKKDKAERRLAYNEEQIHKFDKQKLYYHATKAMSH 600
       ........M.....................................T.

       FSEECVRKYEAFKDKSEEWMRKMLHLRKQLLSLTNQCFDIEEEVSKYQDY 650
       .TD...K.....LN.....I.............................E.

       TNELQETLPQKMLAASGGVKHAMAPIYPSSNTLVEMTLGMKKLKEEMEGV 700
       ............FT..S.I..T.T.........................

       VKELAENNHILERFGSLTMDGGLRNVDCL                      729
       ............................
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B

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TBK1      9   WLLSDILGQGATANVFRGRHKKTGDLYAVKVFNNISFLRPVDVQMREFEVLKKLNHKNIV 68
IKKbeta   15  WEMKERLGTGGFGNVIRWHNQATGEQIAIKQCRQELSPKNRNRWCLEIQIMRRLNHPNVV 74
IKKalpha  15  WEMRERLGTGGFGNVSLYQHRELDLKIAIKSCRLELSSKNRERWCHEIQIMKKLDHANVV 74

TBK1      69  KLFAIEEET----TTRHKVLIMEFCPCGSLYTVLEEPSNAYGLPESEFLIVLRDVVGGMN 124
IKKbeta   75  AARDVPEGMQNLAPNDLPLLAMEYCQGGDLRRYLNQFENCCGLREGAVLTLLSDIASALR 134
IKKalpha  75  KACDVPEEL-NFLINDVPLLAMEYCSGGDLRKLLNKPENCCGLKESQILSLLSDIGSGIR 133

TBK1      125 HLRENGIVHRDIKPGNIMRVIGEDGQSVYKLTDFGAARELEDDEQFVSLYGTEEYLHPDM 184
IKKbeta   135 YLHENRIIHRDLKPENIVLQQGEK-RLIHKIIDLGYAKELDQGSLCTSFVGTLQYLAPEL 193
IKKalpha  134 YLHENKIIHRDLKPENIVLQDVG-GKTIHKIIDLGYAKDVDQGSLCTSFVGTLQYLAPEL 192

TBK1      185 YERAVLRKDHQKKYGATVDLWSVGVTFYHAATGSLPFRPFEGPRRNKEVMYKIITGKPSG 244
IKKbeta   194 LE--------QQKYTVTVDYWSFGTLAFECITG---FRPFLPNWQPVQWHSKVRQKSE-- 240
IKKalpha  193 FE--------NKPYTATVDYWSFGTMVFECIAG---YRPFLHHLQPFTWHEKIKKKDP-- 230

TBK1      245 AISGVQKAENGPIDWSGDMPLSCSLSQGLQALLTPVLANILEAD--QEK-----CWGFDQ 297
IKKbeta   241 VDIVVSEDLNGAVKFSSSLPFPNNLNSVLAERLEKWLQLMLMWHPRQRG--TDPQYGPNG 298
IKKalpha  240 KCIFACEEMTGEVRFSSHLPQPNSLCSLIVEPMESWLQLMLNWDPQQRGGPIDLTLKQPR 299

TBK1      298 FFAETSDVLHRMVIHVFSLQHMTAHKIYIHSYNTAAVFHEL---VYKQTKIVSSNQELI 353
IKKbeta   299 CFRALDDILNLKLVHVL---NMVTGTVHTYPVTEDESLQSLKTRIQENTGILETDQELL 354
IKKalpha  300 CFALMDHILNLKIVHIL---NMTSAKIISFLLPCDESLHSLQSRIERETGINTGSQELL 355
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Fig. 4. TBK1 primary structure. (**A**) Predicted amino acid sequence of murine and human TBK1. The kinase domain is underlined and the shortest region identified in the two-hybrid screen is indicated by the overhead bar. (**B**) Region of homology among TBK1, IKKα (Mock *et al*., 1995) and IKKβ (Nakano *et al*., 1998). Tall bars indicate the residues that are identical between TBK1 and either IKKα or IKKβ; short bars indicate the residues that are similar between TBK1 and either IKKα or IKKβ.

TANK 1–168 blocks TBK1-mediated activation of NF-κB

If formation of the TBK1–TANK–TRAF2 ternary complex is required for TBK1 activity, then a TANK variant that can interact with TBK1 but that cannot interact with TRAF2 should behave as a dominant-negative for TBK1. Cheng and Baltimore (1996) delineated the region between residues 168 and 190 of TANK as that required for TRAF2 association. We therefore tested the effect of titrating TANK 1–168 into co-transfections with TBK1. As shown in Figure 7C, TANK 1–168 inhibited the TBK1-dependent stimulation, while full-length TANK did not inhibit (see below, Figure 10A). TANK 1–168 had no activity in the assay when expressed alone (data not shown). These data suggest that stimulation of NF-κB by TBK1 requires formation of a TBK1–TANK–TRAF ternary complex.

TANK 1–190 with TRAF2 activates JNK1 in a TBK1-independent manner

In addition to their effect on NF-κB, TRAFs 2, 5 and 6 have also been shown to activate JNK1, a kinase in the AP-1 pathway (Song *et al*., 1997). We therefore tested whether TANK modulated TRAF-dependent JNK1 activation and whether TBK1 was involved. We transfected Flag-tagged JNK1 in the absence and presence of TANK 1–190 and TRAF2 and performed an IP-kinase assay using GST–cJun 1–179 as substrate. TANK 1–190 did not stimulate JNK1 activity significantly by itself (Figure 8A, lane 3), but did activate JNK1 synergistically when co-transfected with TRAF2 (cf. lanes 1–4). TANK 1–190 enhanced the JNK1 stimulation observed with TRAF5 and TRAF6 to a similar extent (data not shown). Interestingly, TBK1 K38A did not inhibit the synergistic activation of JNK1 observed with TRAF2 and TANK 1–190 (Figure 8A, cf. lanes 4–6). Furthermore, titration of TBK1 into this assay did not result in reproducible JNK1 activation (Figure 8B). These data indicate that TANK can serve as a cofactor for TRAF-mediated activation of JNK1, but that TBK1 is not involved and is a specific activator of the NF-κB pathway. Another protein may mediate JNK1 activation by TANK and TRAF2.

Dominant-negative TBK1 does not block NF-κB activation by TNFα, IL-1 or CD40

Cheng and Baltimore (1996) showed that the C-terminus of TANK was able to inhibit *in trans* the stimulation of NF-κB by CD40 and CD40 ligand. Since TANK contained stimulatory activity in its N-terminal 190 residues, this led to a model that invoked the involvement of TANK in CD40-mediated activation of NF-κB via the derepression of this inhibitory activity. Other studies, however, concluded that TANK served only an inhibitory role in this pathway since overexpression of full-length TANK inhibited the activation of NF-κB by TNF-α, IL-1, CD40, or by high levels of TRAF2 (Rothe *et al*., 1996). The

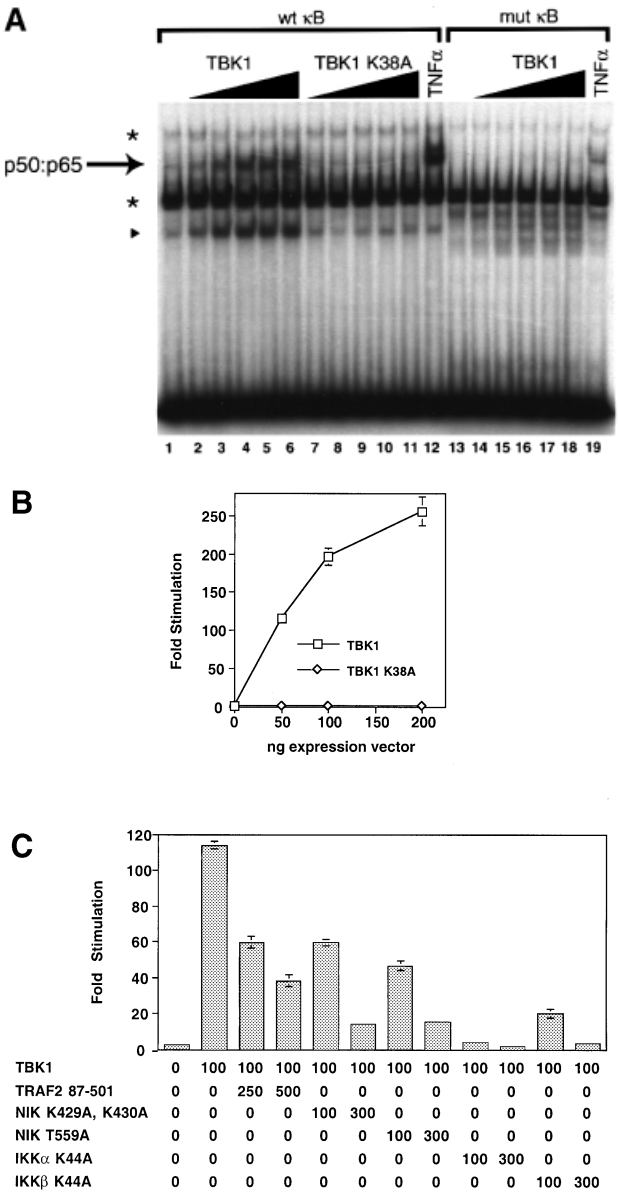

Fig. 5. TBK1 activates NF-κB. (**A**) TBK1 induces κB-specific DNA-binding activity. Expression vectors for TBK1 and TBK1 K38A were titrated into transfections in 293 cells: 50 ng of TBK1 or TBK1 K38A expression vector were used in lanes 2, 7 and 14; 125 ng in lanes 3, 8 and 15; 250 ng in lanes 4, 9 and 16; 500 ng in lanes 5, 10 and 17; and 1000 ng in lanes 6, 11 and 18. Nuclear extracts were prepared 45 h after transfection, and binding reactions were performed as described (Van Antwerp *et al*., 1996) using wild-type (5′-GGGGACTTTCCC-3′, lanes 1–12) or mutant (5′-GGCGACTTTCCC-3′, lanes 13–19) κB site-containing probes. Control extracts were prepared from cells transfected only with empty expression vector (lanes 1 and 13) or by treating 293 cells with TNF-α (10 ng/ml) for 15 min prior to extract preparation (lanes 12 and 19). Supershift analyses revealed that the indicated inducible complex was composed of p50:p65. The composition of the lower inducible complex (small arrow) was not determined; its mobility was not altered by antibodies to p50, p52, p65 or c-Rel. Asterisks indicate non-κB-specific complexes. (**B**) TBK1 activates κB reporter activity. Expression vectors for TBK1 (□) and TBK1 K38A (◇) were titrated into transfections in 293 cells with 100 ng of the $(κB)_3$-IFN-LUC reporter and fold stimulation was determined as in Materials and methods. Points represent the average of at least three experiments. Actual values are for squares: 1.0 ± 0.1; 116 ± 7; 197 ± 11; and 256 ± 19; and for diamonds: 1.00 ± 0.09; 1.12 ± 0.02; 1.02 ± 0.13; and 0.85 ± 0.04. Western blotting of transfected cell extracts confirmed comparable levels of expression of TBK1 and TBK1 K38A. (**C**) Dependence of TBK1 on TRAF2, NIK and the IKK complex. The indicated amounts (in ng) of expression vectors for TBK1 and the indicated dominant-negatives were co-transfected with 100 ng of the $(κB)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 114.0 ± 2.0; 59.5 ± 3.2; 38.2 ± 3.2; 59.4 ± 2.0; 14.0 ± 0.6; 46.5 ± 2.9; 15.0 ± 0.9; 4.0 ± 0.2; 0.8 ± 0.04; 19.8 ± 2.4; and 3.0 ± 0.4.

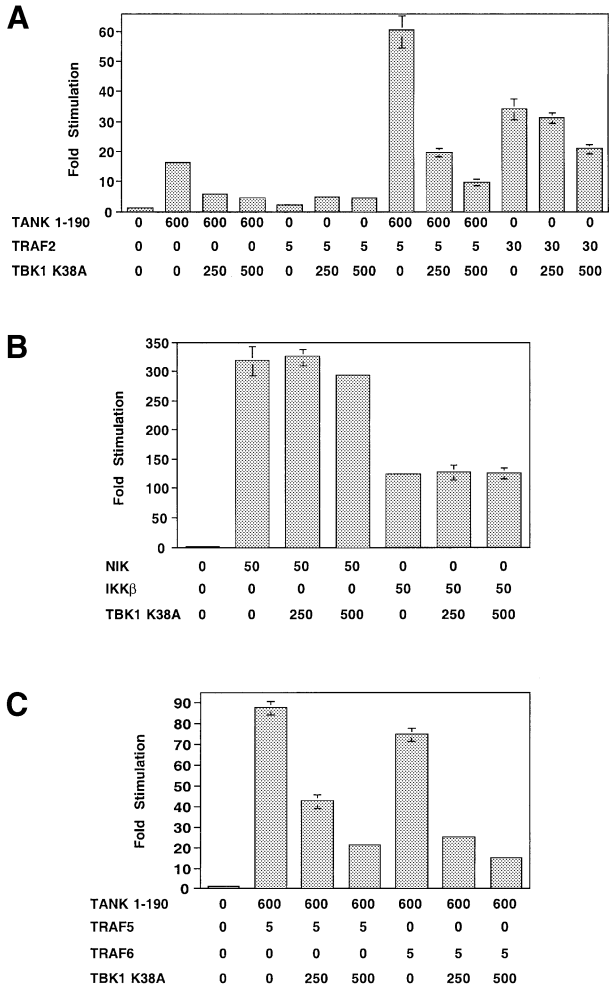

Fig. 6. TBK1 is required for TANK activity, upstream of TRAF2. (**A**) The indicated amounts (in ng) of expression vectors for TANK1–190, TRAF2 and TBK1 K38A were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 16.3 ± 0.6; 5.8 ± 0.3; 4.4 ± 0.3; 2.2 ± 0.1; 4.9 ± 0.3; 4.5 ± 0.2; 60.5 ± 4.7; 19.5 ± 1.5; 9.6 ± 1.2; 34.0 ± 3.3; 31.1 ± 1.6; and 21.1 ± 1.2. (**B**) The indicated amounts (in ng) of expression vectors for NIK, IKKβ and TBK1 K38A were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 318.0 ± 25.0; 326.0 ± 12.0; 294.0 ± 4.0; 125.0 ± 5.0; 128.0 ± 11.0; and 126.0 ± 8.0. (**C**) The indicated amounts (in ng) of expression vectors for TANK1–190, TRAF5, TRAF6 and TBK1 K38A were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 87.5 ± 3.0; 42.7 ± 2.9; 21.1 ± 0.4; 74.5 ± 2.9; 25.0 ± 0.9; and 15.0 ± 1.0.

identification of TBK1 afforded us the ability to re-examine the role of TANK in this pathway and in TNF-α- and IL-1-mediated activation of NF-κB. We titrated the TBK1 K38A mutant into transfections in which the κB-dependent reporter was stimulated by treatment with TNF-α or IL-1, or by co-transfection of CD40 and CD40L (Figure 9A–C). TBK1 K38A did not significantly inhibit any of these stimuli in NF-κB activation. In addition, TBK1 K38A did not affect the stimulation of this reporter

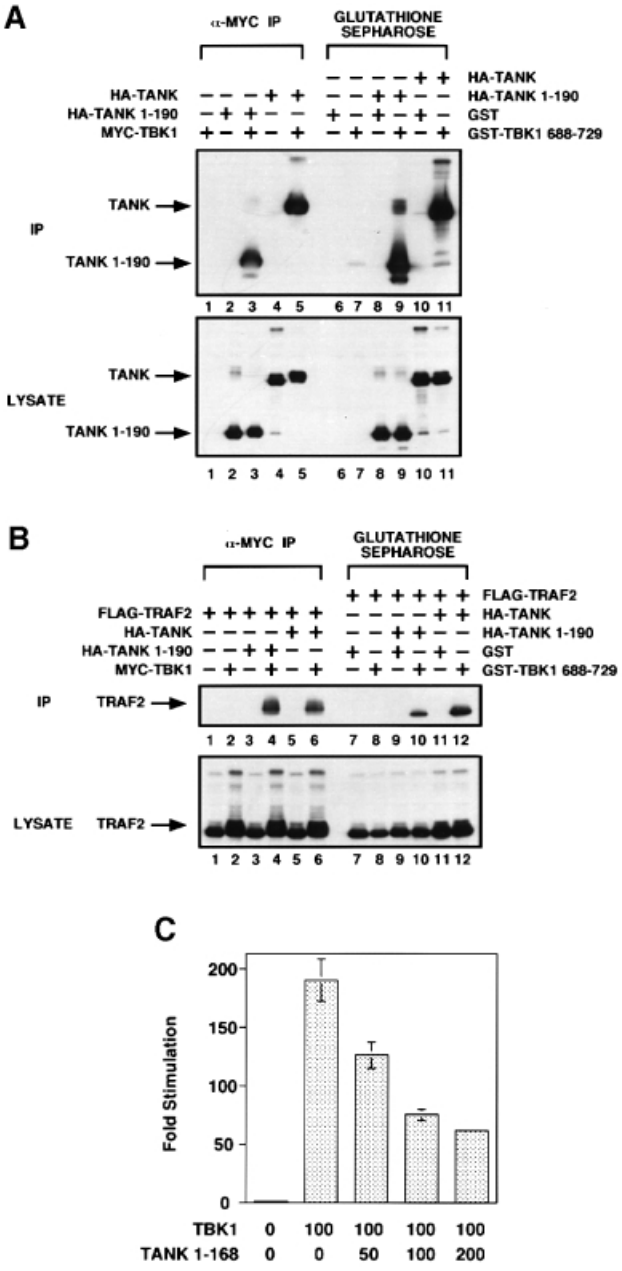

Fig. 7. Association between TANK, TRAF2 and TBK1. (**A**) 293 cells were transfected with expression vectors (500 ng each) for TANK, TANK 1–190, TBK1, GST or GST–TBK1 688–729 as indicated, and α-Myc immunoprecipitation (lanes 1–5) or precipitation with glutathione–Sepharose (lanes 6–11) was performed as described in Materials and methods. The top panel shows the contents of the precipitate, the lower panel shows the lysate (1.25% input), developed with α-HA primary. (**B**) 293 cells were transfected with expression vectors (2 μg each) for TRAF2, TANK, TANK 1–190, TBK1, GST or GST–TBK1 688–729 as indicated, and α-Myc immunoprecipitation (lanes 1–6) or precipitation with glutathione–Sepharose (lanes 7–12) was performed as described in Materials and methods. The top panel shows the contents of the precipitate, the lower panel shows the lysate (1.25% input), developed with α-Flag primary. (**C**) The indicated amounts (in ng) of expression vectors for TBK1 and TANK1–168 were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.2; 190.0 ± 18.0; 126.0 ± 11.0; 74.6 ± 5.2; and 61.6 ± 3.1.

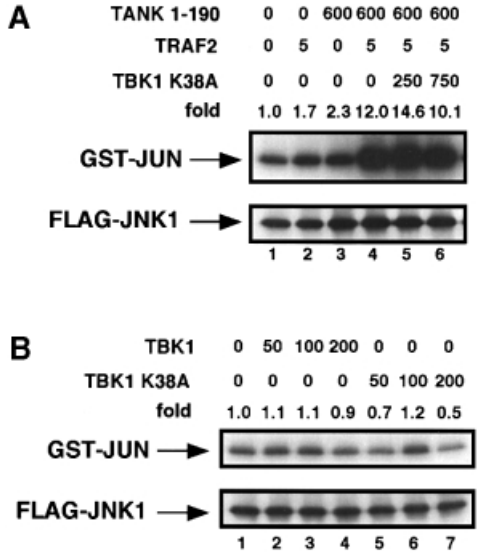

Fig. 8. TANK 1–190 and TRAF2 synergistically activate JNK1 in a TBK1-independent manner. (**A**) The indicated amounts (in ng) of expression vectors for TANK 1–190, TRAF2 and TBK1 K38A were co-transfected into 293 cells with 250 ng of the Flag-JNK1 expression vector, and IP-kinase assays were performed as described in Materials and methods using 500 ng of GST–cJun (1–79) substrate. The amount of radioactivity incorporated into substrate (top panel) was quantitated on a PhosphorImager, and fold indicates the amount incorporated normalized to that observed with Flag-JNK1 alone. The lower panel shows a Western blot with α-Flag primary to indicate the relative amounts of JNK1 kinase in each sample. (**B**) IP-kinase assay for Flag-JNK1 activity as in (A) after co-transfection of 293 cells with the indicated amounts (in ng) of expression vectors for TBK1 or TBK1 K38A with 250 ng of Flag-JNK1 expression vector.

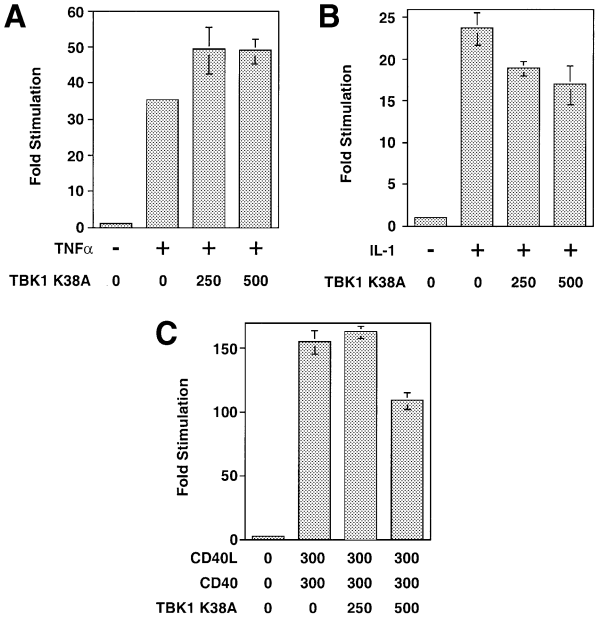

Fig. 9. TBK1 is not required for the activation of NF-κB by TNF-α, IL-1 or CD40/CD40L. (**A**) 293 cells were co-transfected with the indicated amount (in ng) of expression vectors for TBK1 K38A and 100 ng of the $(\kappa B)_3$-IFN-LUC reporter. Approximately 44 h after transfection, the indicated samples were treated with TNF-α (50 ng/ml) for 5 h and then harvested. Fold stimulation was determined as described in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 35.4 ± 0.5; 49.4 ± 6.1; and 49.1 ± 3.0. (**B**) 293 IL-1R1 cells were transfected as in (A), except that they were treated with IL-1 (1 ng/ml) for 5 h before harvest. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 23.7 ± 1.8; 18.9 ± 0.8; and 17.0 ± 2.2. (**C**) 293 cells were co-transfected with the indicated amounts of expression vectors for CD40, CD40L and TBK1 K38A with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter, and fold stimulation was determined as described in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 155.0 ± 9.0; 163 ± 4.0; and 109 ± 6.0.

observed with an activated Toll-like receptor 4 construct in 293 cells, with daunorubicin in 293 cells or with phorbol 12-myristate 13-acetate (PMA)/ionomycin or CD3/CD28 co-stimulation in Jurkat T cells (data not shown). These data argue against a role for TANK and TBK1 in the activation of NF-κB by TNF-α, IL-1, CD40L, LPS, daunorubicin, PMA/ionomycin or CD3/CD28 and suggest that TANK only serves as an inhibitor in the TNF-α, IL-1 and CD40 pathways. The stimulatory, TBK1-dependent role of TANK in NF-κB activation must function in a distinct pathway that converges with other, TBK1-independent pathways at the level of TRAFs.

TANK can simultaneously inhibit CD40 signaling and allow TBK1-mediated activation of NF-κB

If TBK1 functions with TANK in an alternative pathway that activates NF-κB, TBK1 must be resistant to the inhibitory influence of the C-terminal domain of TANK. Indeed, as shown in Figure 10A, titration of full-length TANK had no effect on TBK1-mediated κB reporter stimulation (compare this with the effect of full-length TANK on TRAF2 stimulation in Figure 1C). This is consistent with the results of the immunoprecipitation experiments (Figure 7), which showed that the presence of the C-terminal domain of TANK does not affect the association of TANK with TBK1, or the formation of the TBK1–TANK–TRAF2 complex.

The dual potential of TANK to be inhibitory in TBK1-independent pathways and stimulatory in the TBK1-dependent pathway may allow it to function as a two-way stopcock, able to relay signals from one pathway (TBK1-dependent pathway) while inhibiting signals emanating from receptors for TNF-α, IL-1 or CD40L (TBK1-independent pathway). The experiment shown in Figure 10B highlights this dual activity of TANK. TANK inhibited the stimulation observed with CD40 and CD40L co-transfection, presumably by binding to endogenous TRAFs and preventing their association with the CD40 cytoplasmic tail. Titration of TBK1 under these TANK-inhibitory conditions still resulted in robust stimulation of the κB-dependent reporter. Since TBK1 activity is dependent on TANK and TRAFs, these data demonstrate that while the TANK–TRAF complex is inhibitory for CD40 signaling, it is fully competent for TBK1-mediated activation of NF-κB.

Discussion

We have cloned a novel kinase, TBK1, which appears to mediate the ability of TANK to activate NF-κB. TBK1 binds directly to the stimulatory region of TANK (1–190), can activate NF-κB when overexpressed, and both TBK1- and TANK-dependent stimulation of the κB reporter require TRAF2, NIK and the IKK complex. In addition, kinase-dead TBK1 inhibits TANK-mediated activation of NF-κB and synergy with TRAFs 2, 5 and 6.

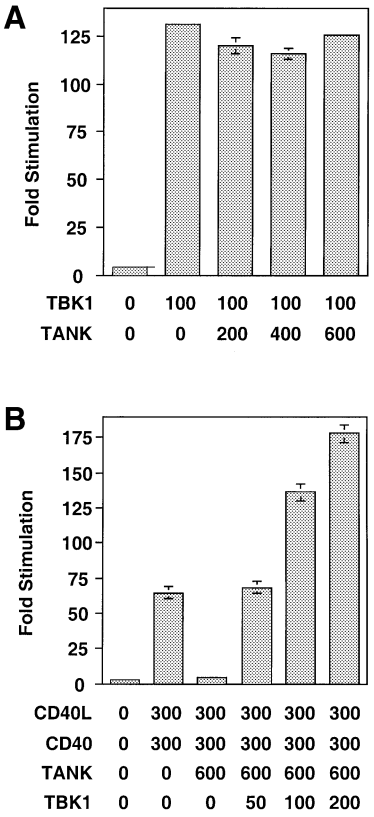

Fig. 10. TBK1 is resistant to the inhibitory activity of TANK. (**A**) The indicated amounts (in ng) of expression vectors for TANK and TBK1 were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as described in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 131.0 ± 2.0; 120.0 ± 4.0; 116.0 ± 3.0; and 126.0 ± 01.0. (**B**) The indicated amounts (in ng) of expression vectors for CD40, CD40L, TANK and TBK1 were co-transfected with 100 ng of the $(\kappa B)_3$-IFN-LUC reporter into 293 cells, and fold stimulation determined as in Materials and methods. The bars represent the average of at least three experiments. Actual values reading from left to right are: 1.0 ± 0.1; 64.5 ± 4.1; 4.1 ± 0.4; 68.3 ± 4.2; 136.0 ± 6.0; and 178.0 ± 6.0.

Initial studies of TANK described both stimulatory and inhibitory effects of TANK on TRAF-mediated NF-κB activation. It was straightforward to imagine how TANK could function as an inhibitor for pathways downstream of TNF-α, IL-1 and CD40 because it was clear that TANK could compete with receptor tails for binding to TRAFs. High concentrations of TANK could therefore prevent the ligand-dependent recruitment of TRAFs to the receptor complex and their subsequent ability to mediate the activation of NIK. In addition, the C-terminal domain of TANK displayed inhibitory activity that was enigmatic but probably involved in the inhibition of TRAF signaling. What remained totally unclear was how TANK could also work as a positive TRAF cofactor, stimulating the ability of TRAF2 to activate NF-κB, and how this stimulatory activity could fit within models of the known signaling pathways.

The identification and initial characterization of TBK1 allow us to conclude that the potential role for TANK in the TNF-α, IL-1 and CD40 pathways is only inhibitory. TBK1 mediates TANK's stimulatory activity and TBK1 is not required for activation of NF-κB by these pathways. The observed stimulatory activity of TANK in these transfection experiments must reflect its role as an activator in a separate pathway that is TBK1-dependent. This activity of TANK probably results from its ability to bridge an association between TBK1 and TRAF2. TANK can interact simultaneously with TBK1 and TRAF2, and formation of the TBK1–TANK–TRAF2 complex appears to be required for signaling, since TANK activity can be inhibited by kinase-dead TBK1, TBK1 activity can be inhibited by TANK 1–168 (which can bind TBK1 but not TRAF2), and both TANK and TBK1 activities can be inhibited by the RING finger-deleted variant of TRAF2.

We have characterized the functional potential of TBK1 by transient transfection. It will be critical to examine the regulation of TBK1 and TBK1-containing complexes at endogenous levels. We suspect that an as yet undetermined physiological stimulus activates NF-κB by initiating the formation of an active TBK1–TANK–TRAF2 signaling complex. TANK and TRAF2 have been shown to be associated in unstimulated 293 cells (Rothe *et al.*, 1996). It is possible that the putative stimulus causes the recruitment of TBK1 to a TANK–TRAF2 complex, either by increasing the associative properties of an existing pool of TBK1 or by up-regulating the concentration of TBK1, for example through transcriptional induction of the TBK1 gene. It is also possible that TBK1 is pre-associated with TANK and TRAF2 in a latent form and that TBK1 kinase activity is activated within the preformed complex as a result of the stimulus. We are actively pursuing the identification of TBK1-dependent stimuli. As discussed above, in the TNF-α, IL-1 and CD40 pathways, TRAFs are recruited into the ligand-bound receptor complex. This recruitment drives subsequent signaling, although it is unclear precisely how receptor complex formation causes the TRAF-mediated activation of NIK. The TBK1–TANK–TRAF2 complex may represent an alternative, non-receptor-bound complex through which TRAFs are activated for subsequent signaling. It is tempting to speculate that the TBK1–TANK–TRAF2 complex may mediate signals emanating from a cell surface receptor (or class of receptors) that cannot recruit TRAFs to its receptor complexes and must use TANK and TBK1 to link into the TRAF–NIK–IKK cascade. Alternatively, the TBK1–TANK–TRAF2 complex may convey intracellular signals that do not depend on extracellular ligands.

The kinase activity of TBK1 promotes signaling dependent upon the TRAF2 RING finger, NIK kinase activity and IKK kinase activity, presumably by stimulating the TRAF2-dependent activation of NIK. We did not detect an association of TBK1 with NIK, nor an association between NIK and the TBK1–TANK–TRAF2 complex, so we do not suppose that TBK1 activates NIK directly. It is intriguing that TBK1 activity results in the phosphorylation of TRAF2 and TANK, but further experiments are needed to determine whether these events are required for TBK1 signaling or are merely markers of TBK1 activity. The identification of the direct substrate(s) for TBK1 will be required to determine the exact mechanism of TBK1 action. Based on its homology to IKKα and IKKβ, one might have predicted TBK1 to be an IκB kinase. It is clear, however, that TBK1 functions further upstream; TBK1 activity is blocked by dominant-negative versions of TANK, TRAF2, NIK, IKKα and IKKβ. While one must be generally cautious in interpreting the effects

of dominant-negatives, we note that dominant-negative variants of TBK1, TRAF2, NIK and IKKα or IKKβ all display clear epistatic relationships to one another, allowing an ordering of the functional potential of each component of the pathway.

While we have done most of our characterization of TANK and TBK1 using TRAF2, it appears that TANK and TBK1 can operate on multiple TRAFs. TANK can inhibit TBK1-independent pathways that depend upon different TRAFs for signaling (e.g. TNF-α and IL-1), and can synergize in a TBK1-dependent manner with TRAFs 2, 5 and 6 in the activation of NF-κB. A binding motif in the central region of TANK contains the PXQXS/T TRAF-binding consensus also found in CD40, CD30, CD27 and LMP1 (Arch *et al*., 1998). We also note the presence of a DEED motif in TANK (residues 218–221), which resembles the short acidic TRAF-binding stretches found in 4-1BB, Ox40 and CD30 (Arch *et al*., 1998). While TANK has been shown to bind directly to TRAFs 1, 2 and 3 (Cheng and Baltimore, 1996; Rothe *et al*., 1996), it may also associate with other TRAFs either through direct binding or by interacting with one component of a TRAF heteromultimer.

As indicated above, the C-terminal domain of TANK (190–413) mediates an inhibitory activity that remains enigmatic. TANK 1–190 and full-length TANK activate NF-κB when overexpressed, presumably by promoting the association of endogenous TBK1 and TRAF2. That TANK 1–190 is a more robust activator of NF-κB than full-length TANK implies that the C-terminal domain can oppose TBK1 activity. However, we have also demonstrated that overexpressed TBK1 is resistant to the inhibitory effects of full-length TANK. It is possible that signaling through the TBK1–TANK–TRAF2 complex is regulated by a balance between TBK1 kinase activity, which docks to the TANK N-terminus, and an inhibitory activity that may dock to the C-terminus. Overexpression of TBK1 may shift the balance toward stimulation. Full-length TANK may show a biphasic profile of activation (Cheng and Baltimore, 1996; Figure 1A) because endogenous TBK1 may be recruited to TANK at lower TANK concentrations than the putative C-terminus-associated activity, but once the inhibitory activity is recruited it can block the endogenous TBK1 activity. TANK 1–190 may be more robust because it can not recruit this putative inhibitory activity. A natural alternatively spliced variant of TANK, TANK/I-TRAFγ (Rothe *et al*., 1996), lacks the C-terminal domain and may be an especially potent activator of NF-κB.

Interestingly, in addition to its effects on NF-κB activation, TANK also appears to synergize with TRAFs in the activation of JNK activity, although this does not involve TBK1. It is possible that there is a TBK1 analog in the pathway leading to JNK that associates with TANK and TRAF2 in a similar fashion. It will be of interest to determine whether stimuli that activate NF-κB through TBK1 also activate JNK activity through TANK and this putative unidentified factor. Alternatively, there might be stimuli that are specific in activating either NF-κB or JNK through the use of TANK and selective activation of its associated factors.

TBK1 and TANK are widely expressed proteins, and may regulate NF-κB activation in multiple tissues. This initial characterization of TBK1 in cell lines provides a useful starting point for an assessment of the physiological importance of TBK1-dependent activation of NF-κB, which awaits the characterization of mice deficient in TANK and TBK1.

While this manuscript was in preparation, we became aware of IKK-i, a cytokine-inducible kinase that is 48% identical, 64% similar to TBK1 (Shimada *et al*., 1999). IKK-i can activate NF-κB and JNK and appears to act upstream of TRAF2, also through direct interaction with TANK (T.Kawai, F.Nomura and S.Akira, personal communication). IKK-i may be responsible for the TANK-mediated activation of JNK. Thus, TBK1 and IKK-i represent a new family of IKK-related kinases that promote signaling downstream of TRAFs by docking to TANK.

Materials and methods

Transfections, cell culture and NF-κB reporter assays

Both 293 and 293 IL-1R1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 U/ml each of penicillin and streptomycin, and 2 mM glutamine in humidified 5% CO_2 at 37°C. Cells were plated at 5×10^5/well in 6-well dishes 24 h before transfection by the calcium phosphate method using a total of 2 μg of DNA. The medium was changed 20–24 h after transfection, and at 42–48 h after transfection cells were washed with phosphate-buffered saline (PBS) and lysed in 250 μl of reporter lysis buffer (Promega) on ice for 10 min. Cell debris was removed by centrifugation at 13 000 r.p.m. at 4°C for 5 min. A 10 μl aliquot of extract was used to measure luciferase activity using the Luciferase Assay System (Promega) and a luminometer (Optocomp I, MGM Instruments) integrating for 10 s with no delay according to the manufacturer's instructions. TNF-α (recombinant human TNF-α, Genzyme Diagnostics) and IL-1 (recombinant human IL-1α, R&D Systems Inc.) were added 5 h before harvest. The $(\kappa B)_3$-IFN-LUC reporter has been described (Cheng and Baltimore, 1996). For normalization to transfection efficiency, each transfection included 10 ng of pCSK-LacZ, which constitutively expresses β-galactosidase (Condie *et al*., 1990). β-galactosidase activity was determined using 25 μl of extract and the chemiluminescent β-Gal Reporter Gene Assay (Boehringer Mannheim) according to the manufacturer's instructions. In each experiment, each sample was supplemented with the appropriate empty parental expression vector(s) so as to keep the total amount of each type of vector constant in each sample (e.g. all pcDNA3-derived vectors). The total DNA content in each transfection was made 2 μg by supplementation with pBSK (Stratagene). Fold stimulation was calculated for each sample by dividing the luciferase activity, normalized to β-gal activity, by that observed in the sample containing only empty parental expression vectors.

Electrophoretic mobility shift assay

Mobility shift assays were done essentially as described (Van Antwerp *et al*., 1996) using nuclear extracts prepared from 293 cells transfected as described above except that 2×10^6 cells were plated 24 h before transfection and a total of 10 μg of DNA were used. DNA probes containing wild-type and mutant κB sites were sc-2505 and sc-2511, respectively, obtained from Santa Cruz Biotechnology.

Expression constructs

To make TANK expression constructs for full-length TANK (pc-TANK), TANK 1–190 (pc-TANK1-190) or TANK 1–168 (pc-TANK1-168), the appropriate region of TANK (Cheng and Baltimore, 1996) was amplified by PCR and cloned into pcDNA3 (Invitrogen) so as to encode an HA epitope (YPYDVPDYA) at the C-terminus. The TBK1 expression construct, pc-TBK1, was generated by cloning the murine cDNA into pcDNA3 so as to encode a c-Myc epitope (EQKLISEEDL) and a His_6 tag at the N-terminus of the protein. The pc-TBK1 K38A vector is identical to pc-TBK1, except that the codon for Lys38 was changed to one for alanine by PCR-based mutagenesis. To make the GST–TBK1 688–729 expression vector, the region encoding TBK1 residues 688–729 was PCR-amplified and cloned into pEBG to make an in-frame fusion at the C-terminus of GST. The parental pEBG vector expresses GST under the control of the EF1α promoter.

Expression vectors for wild-type TRAF2 and the 87–501 variant have been described (Rothe *et al*., 1995), as have those expressing TRAF6 (Cao *et al*., 1996), wild-type and KK429–430AA NIK (Malinin *et al*., 1997), NIK T559A (Lin *et al*., 1998), IKKβ (Woronicz *et al*., 1997) and CD40L (Cheng and Baltimore, 1996). The TRAF5 expression vector contains TRAF5 in pRK5 with an N-terminal Flag tag (gift of X.Yang). Expression vectors for wild-type IKKα and the K44A mutant were obtained from X.Yang and I.Stancovski and encode IKKα with an N-terminal Flag tag in the pRK5 vector. The IKKβ K44A mutation was introduced by PCR-based mutagenesis (gift of I.Stancovski). The expression vector for CD40 encodes CD40 in the pcD-x vector (gift of R.Khosravi-Far).

JNK assays

293 cells were transfected by the calcium phosphate method as described above using a total of 2 μg of DNA. Each transfection included 250 ng of pCMV-M2-JNK1 (Yang *et al*., 1997), which expresses Flag-tagged JNK1. At 40–44 h post-transfection, cells were washed with PBS and then lysed in 500 μl of IP-kinase lysis buffer [20 mM Tris pH 7.5, 140 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM *p*-nitrophenyl phosphate, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF)] on ice for 10 min. Cell debris was removed by centrifugation at 13 000 r.p.m. at 4°C for 10 min. To each lysate, 3 μg of M2 (α-Flag) monoclonal antibody (Eastman Kodak) were added and samples were rotated at 4°C for 1 h. Subsequently, a 15 μl bed of protein G–Sepharose (Pharmacia) was added and samples were rotated at 4°C for 1 h. Beads were washed twice with 1 ml of lysis buffer, followed by two washes with 1 ml of reaction buffer (25 mM HEPES pH 7.9, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM *p*-nitrophenyl phosphate, 0.1 mM sodium orthovanadate). Kinase reactions were initiated by adding to each sample of beads 30 μl of reaction buffer plus 50 μM ATP, 5 μCi of [γ-^{32}P]ATP and 0.5 μg of GST–cJun (1–79) (Santa Cruz). After 20 min at 30°C, reactions were terminated with SDS–PAGE loading buffer. Reactions were resolved on 12% SDS–PAGE gels, transferred to Immobilon P (Millipore) membranes and then exposed for autoradiography and quantitation using a Storm 860 PhosphorImager (Molecular Dynamics) and ImageQuant 4.2 software. Subsequent to quantitation of radioactivity, membranes were Western blotted using polyclonal rabbit α-Flag antibodies (Santa Cruz) as primary to visualize the relative amount of Flag-JNK1 in each kinase reaction.

Two-hybrid screening and cDNA cloning of TBK1

Residues 1–190 of TANK were cloned in-frame, N-terminal to the GAL4 DNA-binding domain, into the bait vector pYHL3 (gift of Y.H.Li and X.Yang) to produce pYHL-TANK1-190. pYHL-TANK1-190 was transformed into the yeast strain Y190, and this strain was transformed with a human B-cell plasmid cDNA library in pACTII (Cheng and Baltimore, 1996) and selected on yeast synthetic media lacking histidine, leucine and tryptophan, but containing 25 mM 3-aminotriazole. Colonies surviving after 4–7 days were tested for β-gal activity (Yang *et al*., 1997) and plasmid DNA was prepared from positive colonies and sequenced for identification of cDNA clones.

The cDNA for human TBK1 was cloned by PCR screening of an arrayed human spleen cDNA library (OriGene Technologies) according to the manufacturer's instructions using primers designed from the two-hybrid clones. The murine cDNA was cloned by conventional screening of a murine spleen cDNA λ phage library (Stratagene) using a probe derived from the human cDNA. Full-length clones were sequenced with an automated sequencer (Applied Biosystems). The murine cDNA contains an in-frame stop codon upstream of the first ATG in the predicted open reading frame. Sequence data have been submitted to the DDBJ/EMBL/GenBank database under the accession Nos AF191838 and AF191839 for human and murine TBK1, respectively.

Immunoprecipitations and glutathione–Sepharose precipitations

293 cells were transfected by the calcium phosphate method as described above except that the immunoprecipitations in Figure 7B were scaled up: 2×10^6 cells were plated 24 h before transfection, and a total of 10 μg of DNA were used. At 48 h post-transfection, cells were washed with PBS and then lysed in 0.5 ml (1 ml for Figure 6B) of IP lysis buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% NP-40, 10% glycerol) on ice for 10 min. Cell debris was removed by centrifugation at 13 000 r.p.m. at 4°C for 10 min. For α-myc immunoprecipitation, to 400 μl of each lysate were added 5 μl of anti-c-Myc–agarose conjugate (Santa Cruz) and rotated at 4°C for 1 h. For GST pull-downs, to 400 μl of each lysate was added a 10 μl bed of glutathione–Sepharose (Pharmacia) and rotated at 4°C for 1 h. Beads were washed four times with 1 ml of IP lysis buffer, boiled in the presence of SDS–PAGE loading buffer, resolved on 10–12% SDS–PAGE gels, and then transferred to Immobilon P membranes for Western blotting with α-HA (rabbit polyclonal, Santa Cruz) or α-Flag (rabbit polyclonal, Santa Cruz) antibodies.

Acknowledgements

We thank members of the Baltimore laboratory for helpful discussions and support, I.Stancovski, X.Yang, Y.H.Li, G.Cohen, B.Chen, R.Khosravi-Far, Z.Cao, D.Goeddell, W.Greene, C.Janeway and D.Wallach for reagents, and A.Greenwood, J.Kim, L.Van Parijs, D.Van Antwerp, C.Lois and I.Stancovski for critical reading of the manuscript. We also thank G.Cheng, T.Kawai, S.Akira, R.Peters and T.Maniatis for communication of results prior to publication. J.L.P. is supported by a postdoctoral fellowship from the Helen Hay Whitney Foundation. This work was supported by the NIH grant AI42549-02.

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Received July 28, 1999; revised September 30, 1999; accepted October 4, 1999