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Association of the Adaptor TANK with the IκB Kinase (IKK) Regulator NEMO Connects IKK Complexes with IKKε and TBK1 Kinases*

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Canonical activation of NF-κB is mediated via phosphorylation of the inhibitory IκB proteins by the IκB kinase complex (IKK). IKK is composed of a heterodimer of the catalytic IKKα and IKKβ subunits and a presumed regulatory protein termed NEMO (NF-κB essential modulator) or IKKγ. NEMO/IKKγ is indispensable for activation of the IKKs in response to many signals, but its mechanism of action remains unclear. Here we identify TANK (TRAF family member-associated NF-κB activator) as a NEMO/IKKγ-interacting protein via yeast two-hybrid analyses. This interaction is confirmed in mammalian cells, and the domains required are mapped. TANK was previously shown to assist NF-κB activation in a complex with TANK-binding kinase 1 (TBK1) or IKKε, two kinases distantly related to IKKα/β, but the underlying mechanisms remained unknown. Here we show that TBK1 and IKKε synergize with TANK to promote interaction with the IKKs. The TANK binding domain within NEMO/IKKγ is required for proper functioning of this IKK subunit. These results indicate that TANK can synergize with IKKε or TBK1 to link them to IKK complexes, where the two kinases may modulate aspects of NF-κB activation.

NF-κB transcription factors function as critical mediators of numerous signals during immune, inflammatory, and stress responses. These factors transcriptionally induce many genes whose products are critical to drive immune responses in general or to fight pathogens directly (1–4). In addition, NF-κB is also directly involved in growth and survival of cells relevant in stress and immune responses, due to its antiapoptotic and proliferation-promoting functions (4). A wide variety of extracellular signals initiate signaling cascades that culminate in the phosphorylation and subsequent proteolytic degradation of NF-κB-inhibitory proteins collectively termed IκBs (5). Degradation of the inhibitors liberates the previously bound NF-κB proteins to localize to the nucleus and bind to so-called κB DNA binding elements located within many promoters/enhancers. The IκB inhibitors are phosphorylated on specific serine residues (5, 6) by kinases residing in a large complex referred to as the IκB kinase complex (IKK).[1] IKKs are composed of two catalytic subunits, IKKα and IKKβ (7–11), as well as a regulatory protein, named NEMO (NF-κB essential modulator)/IKKγ/FIP-3 (12–14). More recently, it has been demonstrated that the IKK kinases target not only the so-called small IκB inhibitors, of which the IκBα is the prototype, but that they also similarly phosphorylate and regulate the p105/NF-κB1 and p100/NF-κB2 precursors, leading either to their proteolytic degradation or to their processing to p50 and p52, respectively (15–18). In addition to these functions, IKK kinase activity may also modulate the transactivation potential of the NF-κB proteins liberated by the degradation of the inhibitors; activated IKK kinases have been shown to phosphorylate a transactivation domain of RelA, thereby promoting its ability to transcriptionally transactivate genes (19).

NEMO/IKKγ is an essential component of the IKK complex, as evidenced for example by the inability of many signals, including TNF and interleukin-1, to induce NF-κB activity in NEMO/IKKγ-deficient cells (13, 20, 21). It has been suggested that NEMO/IKKγ may be required for the correct assembly of the IKK complex and/or for the recruitment of upstream activators of the IKK complex (12, 13). However, the functions and mechanisms of NEMO/IKKγ remain to be determined. If this essential component does indeed connect to a variety of different upstream signaling mediators, these would be important to identify, since they may be signal-specific mediators of NF-κB activation and thus more specific potential targets for therapies intended to delimit NF-κB activation.

We have used NEMO/IKKγ as bait in a yeast two-hybrid screening to identify potential mediators of select upstream signaling pathways. Previously, we reported on the identification of one NEMO/IKKγ-interacting protein identified in this way and termed CIKS (connection to IKK and SAPK/JNK) (22) (also known as Act-1 (23)). Here we describe the identification of an additional NEMO/IKKγ-interacting protein, termed TANK (TRAF family member-associated NF-κB activator). We show that TANK interacts with NEMO/IKKγ (and the IKKs) in mammalian cells. TANK had previously been shown to be

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[1] The abbreviations used are: IKK, IκB kinase; TBK1, TANK-binding kinase 1; TNF, tumor necrosis factor; GST, glutathione *S*-transferase; HA, hemagglutinin; aa, amino acid(s); PMA, phorbol 12-myristate 13-acetate; P/I, PMA and ionomycin.

potentially involved in both positive and negative regulation of NF-κB activity (24–26). Positive regulation reportedly occurs via an association of TANK with two kinases, termed inducible IκB kinase (also known as IKKε) and TBK1 (also known as T2K and NF-κB-activating kinase) (27–29), although the mechanisms involved remain unknown. We demonstrate here that TANK synergizes with IKKε and TBK1 to form a complex with NEMO/IKKγ and thus with the IKKs. This links IKKε and TBK1 with at least a subset of IKK complexes and suggests potentially direct effects on IKK-associated functions. We also provide evidence that the TANK-binding domain of NEMO may be important in transmitting signals.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—Human embryonic kidney 293 and HeLa cells were maintained as described (22, 30). NEMO-deficient Jurkat cells were a generous gift from Dr. Shao-Cong Sun, (Pennsylvania State University College of Medicine) and were maintained in RPMI supplemented with 10% fetal bovine serum and 10% penicillin/streptomycin.

Polyclonal anti-TANK rabbit antibodies were raised against the first 20 and the last 19 amino acids of human TANK. Anti-NEMO/IKKγ and anti-Myc antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), as were anti-HA and anti-IKKα beads. Anti-FLAG beads were purchased from Sigma. Monoclonal anti-IKKε and anti-IKKα antibodies were from Imgenex (San Diego, CA) and BD PharMingen (San Diego, CA), respectively. Mouse NEMO/IKKγ and human TANK were both cloned by PCR from a mouse or human liver cDNA library, respectively (CLONTECH, Palo Alto, CA). Truncation mutants of NEMO/IKKγ and TANK were generated by PCR. The FLAG-NEMO ΔTANK construct was made by first cloning a PCR-generated fragment encompassing the region from amino acid 250 to the stop codon into pcDNA3.1 FLAG; subsequently, a PCR-generated fragment harboring amino acids 2–200 was inserted in frame. The HA-NEMOΔTANK construct was made by subcloning the NEMOΔTANK coding sequence into pcDNA3.1 HA. The FLAG-TANK ΔIKKε construct was made by cloning a PCR-generated fragment encoding amino acids 2–110 into pcDNA3.1 FLAG ΔN169-TANK. Full-length CIKS, NEMO/IKKγ, IKKα, and IKKβ have been previously described (22, 30). TBK1 and IKKε were PCR-amplified from a liver cDNA library. cDNAs encoding TANK and IKKε were cloned into pcDNA3.1 FLAG (Invitrogen) for expression in mammalian cells, and TBK1 and IKKε were cloned into pcDNA3.1 Myc. Both TBK1 and IKKε mutant (K38A) constructs were generated by site-directed mutagenesis (Stratagene, La Jolla, CA). A construct encoding full-length NEMO/IKKγ fused to GST was generated by subcloning full-length NEMO/IKKγ into pGEX-2T (Amersham Biosciences).

Yeast Two-hybrid Analysis—DNA encoding NEMO/IKKγ (amino acids 1–339) was cloned into the GAL4 DNA-binding vector pGBT9 (CLONTECH, Palo Alto, CA) and used as bait in a two-hybrid screen of a human liver cDNA library (CLONTECH) in *Saccharomyces cerevisiae* Y190; positive clones were selected as described (22).

In Vitro Translation and GST Pull-down Assays—*In vitro* transcription and translation were carried out with 1 μg of HA-TANK, as described (22). Both the full-length GST-NEMO/IKKγ fusion protein and the wild type GST were produced and purified as described (22). Protein-protein interactions were performed by incubating an aliquot of GST-NEMO/IKKγ or GST bound to the glutathione-Sepharose beads with 5 μl of *in vitro* translated HA-TANK as described (31). Beads were washed five times with 1 ml of phosphate-buffered saline, 1% Triton, protease inhibitors; resuspended into migrating buffer; and run on an SDS-polyacrylamide gel before autoradiography.

Immunoprecipitations—For immunoprecipitations involving overexpressed proteins, 293 or HeLa cells (3×10^6) were transfected via LipofectAMINE (Invitrogen) with expression vectors as indicated in Figs. 2 (*B*, *C*, and *D*), 3 (*B* and *D*), and 4 (*B*, *C*, and *D*). 24 h after transfection, cells were then washed with phosphate-buffered saline and lysed in 0.5% Triton lysis buffer. Ectopically expressed proteins were immunoprecipitated by using anti-FLAG or anti-HA antibodies bound to agarose beads or by using anti-NEMO antibodies (as indicated). The immunoprecipitate was washed five times with 0.5% lysis buffer and subjected to SDS-PAGE.

Immunoprecipitations of endogenous NEMO/IKKγ or endogenous IKKα were performed with 293 or HeLa cells (6×10^6) after cells had been transfected with expression vectors for various proteins as indicated in Figs. 5 and 6*B*. Harvested cells were washed and lysed as described above. Anti-NEMO/IKKγ immunoprecipitations were performed by incubating the total cell extracts with polyclonal antibodies for 2 h at 4 °C, followed by an overnight incubation with protein A-agarose conjugate (Santa Cruz Biotechnology) (see Figs. 5, *A* and *C*, and 6), whereas anti-IKKα immunoprecipitations were performed by incubating the cell extracts with monoclonal anti-IKKα beads overnight (see Fig. 5*B*).

For identification of ternary complexes by immunoprecipitation experiments (see Figs. 2*D* and 7 (*A* and *B*)), 293 cells (10^7) were transfected with the indicated expression vectors, as described above. 24 h after transfection, ectopically expressed TANK was immunoprecipitated with anti-FLAG for 2 h at 4 °C. The immunoprecipitate was washed five times with the lysis buffer and incubated overnight with the FLAG peptide (Sigma), according to the protocol provided by the manufacturer. The supernatants were subsequently incubated with anti-NEMO antibodies and protein A-agarose conjugate overnight. The resulting immunoprecipitates were washed with the lysis buffer and subjected to SDS-PAGE.

For endogenous coimmunoprecipitations, 293 cells (6×10^7) were left untreated or were stimulated with 40 ng/ml of PMA and 2 μM ionomycin (Sigma) for the indicated period of time and subsequently lysed in the lysis buffer. An anti-TANK immunoprecipitation was then performed with the polyclonal antibodies for 2 h at 4 °C, followed by incubation overnight with protein A-agarose conjugate. An immunoprecipitation with an aliquot of a prebleed rabbit serum was performed in parallel as a negative control. The immunoprecipitates were subjected to anti-IKKε and -IKKα Western blots.

Reporter Assay—Jurkat NEMO-deficient cells (6×10^6) were transfected in 10-cm dishes using the DMRIE-C reagent (Invitrogen) and 5 μg of the Ig-κB-luciferase reporter (30), with or without 1 μg of the indicated FLAG-NEMO constructs.

RESULTS

Identification of TANK as a NEMO/IKKγ-interacting Protein—To gain insights into how NEMO/IKKγ may transmit signals to the IKKs, we screened for NEMO/IKKγ -interacting proteins via yeast two-hybrid assays. Mouse NEMO/IKKγ (aa 1–339) was used as bait in a fusion with the DNA binding domain of GAL4 to trap interacting proteins generated from a human liver expression library fused to the GAL4 activation domain. Positive clones isolated included portions of IKKα, IKKβ, and CIKS (22). In addition, three independent and overlapping clones encoded parts of TANK (also known as TRAF-interacting protein or I-TRAF). TANK had previously been identified as a potential regulator in NF-κB-activating pathways, although its precise role is controversial. TANK was originally discovered as a protein capable of binding to TRAF1, -2, and -3 (24–26).

To delineate the region in NEMO/IKKγ that is required for interaction with TANK, we tested various truncation mutants of NEMO/IKKγ (fused to the GAL4 DNA binding domain) for binding to the isolated TANK-GAL4 activation domain fusion protein in yeast (Fig. 1*B*). Among the C-terminal deletion mutants of the 412-amino acid-long NEMO/IKKγ protein, one lacking the last 100 amino acids and thus lacking the entire leucine zipper domain was still able to interact with TANK. Among the various N-terminal deletions of NEMO/IKKγ, those lacking any or all of the first 200 amino acids were still able to bind TANK, whereas one lacking the first 250 amino acids was not. Complementing this result, a NEMO/IKKγ construct composed of amino acids 150–250 was sufficient to mediate the interaction with TANK. Based on these findings, we conclude that the region between amino acids 200 and 250 of NEMO/IKKγ mediates binding to TANK. This domain of NEMO/IKKγ is distinct from the one required for interaction with the IKKs (amino acids 50–100) (32, 33).[2] The interaction between NEMO/IKKγ and TANK was also confirmed *in vitro*. An *Escherichia coli*-produced recombinant GST-NEMO/IKKγ fusion protein (full-length) bound *in vitro* translated [^{35}S]HA-TANK,

[2] A. Leonardi and U. Siebenlist, unpublished data.

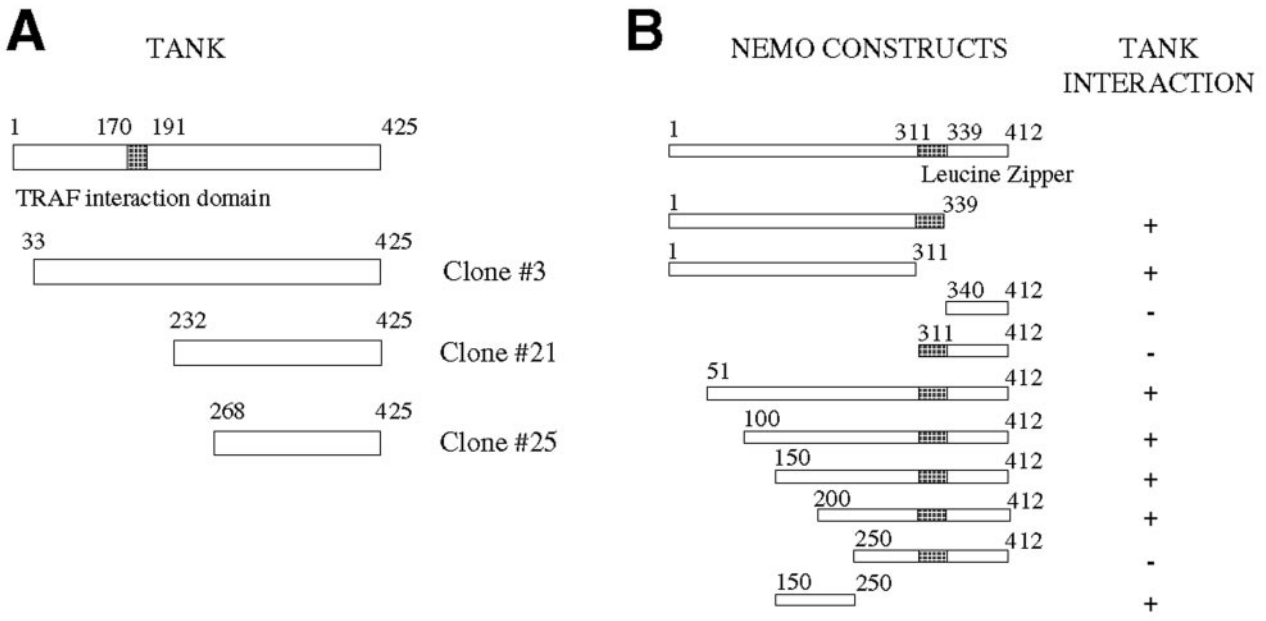

FIG. 1. **NEMO/IKKγ and TANK interact in yeast.** *A*, schematic representation of the human TANK protein and of the three products encoded by the clones isolated by yeast two-hybrid screening. The TRAF interaction domain (aa 170–191) is highlighted. *B*, mapping of the TANK interaction domain on NEMO/IKKγ by yeast two-hybrid experiments. The various NEMO/IKKγ constructs, cloned in frame with the GAL4 DNA binding domain of the pGBT9 vector, are schematically represented. The leucine zipper domain (from aa 311 to 339) of NEMO/IKKγ is marked.

whereas recombinant GST alone did not (Fig. 2*A*, *top panel*).

Next we investigated the interaction of NEMO/IKKγ with TANK in mammalian cells. FLAG-TANK was transiently co-expressed in 293 cells together with HA-NEMO/IKKγ or a NEMO/IKKγ mutant lacking the first 250 amino acids (HA-NEMO/IKKγ ΔN250). Cell extracts were immunoprecipitated with anti-FLAG antibodies (Fig. 2*B*, *top panel*) and HA-NEMO/IKKγ was co-immunoprecipitated, but only if FLAG-TANK had also been co-transfected (*lane 5*). The specificity of the interaction between NEMO/IKKγ and TANK was confirmed by the fact that the NEMO/IKKγ deletion mutant lacking the first 250 amino acids (HA-NEMO/IKKγ ΔN250) was not co-immunoprecipitated with TANK (*lane 6*), in agreement with the data in yeast. Similar results were obtained when the NEMO/IKKγ was immunoprecipitated to look for TANK (data not shown). Note that when both NEMO/IKKγ and TANK were co-expressed, a shift in the migration of the TANK protein was detected by Western blot, even in the absence of any overexpressed kinases (*middle panel*, *lane 5*). To further confirm the interaction between TANK and NEMO/IKKγ, 293 cells were transfected with both HA-NEMO/IKKγ and FLAG-TANK, and extracts were immunoprecipitated with anti-HA (Fig. 2*C*). TANK was co-immunoprecipitated with NEMO/IKKγ (Fig. 2*C*, *top panel*, *lane 2*). A shift in the TANK protein was detected when co-expressed with NEMO/IKKγ (Fig. 2*C*, *middle panel*; *lane 2*, *FLAG-TANK**), most likely due to phosphorylation. Interestingly, it is this slower migrating form of TANK that preferentially co-immunoprecipitated with HA-NEMO/IKKγ (Fig. 2*C*, *top panel*, *lane 2*).

Because FLAG-TANK could also be shown to co-immunoprecipitate with HA-IKKβ, especially if NEMO/IKKγ was cotransfected (data not shown), we asked whether these three proteins might be able to form a ternary complex. To test this, 293 cells were transfected either with HA-IKKβ or with FLAG-TANK or both (Fig. 2*D*). Anti-FLAG immunoprecipitations were carried out, and the immunoprecipitates were released from the beads by incubating them with a FLAG peptide. The released material was immunoprecipitated with antibodies to the endogenous NEMO/IKKγ and an anti-HA Western analysis was performed, revealing the presence of IKKβ (*lane 2*). Therefore, a ternary complex of TANK, NEMO/IKKγ, and IKKβ must have been formed in 293 cells. Importantly, this complex was formed with endogenous NEMO/IKKγ, demonstrating that endogenous levels of NEMO/IKKγ were sufficient to mediate the interaction between transfected TANK and IKKβ.

Two Distinct Regions of TANK Are Required for Interaction with NEMO/IKKγ—To delineate the domain in TANK required for interaction with NEMO/IKKγ in mammalian cells, various truncated TANK proteins were generated (Fig. 3, *A* and *C*) and tested for their ability to co-immunoprecipitate with NEMO/IKKγ in 293 cells (Fig. 3, *B* and *D*). A TANK protein lacking the first N-terminal 30 aa (Fig. 3*A*) was able to co-immunoprecipitate with NEMO/IKKγ (Fig. 3*B*, *lane 7*), but TANK proteins lacking the first N-terminal 70 aa or more were not (*lanes 3–5*, *8*, and *9*). All C-terminal deletions of TANK tested (Fig. 3*C*) failed to co-immunoprecipitate with NEMO/IKKγ (Fig. 3*D*, *lanes 3–8*). This suggests that an N-terminal TANK domain (between aa 30 and 70) and a C-terminal TANK domain (between aa 248 and 425) are both required for interaction with the regulatory subunit of the IKK complex in mammalian cells. (The same results were obtained with a NEMO/IKKγ construct lacking the C-terminal 72 aa; data not shown). By contrast, the C-terminal domain of TANK was sufficient in yeast (see Fig. 1*A*). The reason for this is not clear, but the assay for the interaction in yeast may be more sensitive than the one in mammalian cells.

TANK Binding-deficient NEMO/IKKγ Mutant Impaired in Mediating PMA and Ionomycin (P/I)-induced NF-κB Activation—We next explored the possible relevance of the interaction of TANK with NEMO/IKKγ in mediating activation of NF-κB. A NEMO/IKKγ mutant was constructed in which the TANK-binding domain was specifically deleted (NEMOΔTANK) (Fig. 4*A*). When overexpressed in 293 cells, this NEMO mutant failed to interact with TANK (Fig. 4*B*, *top panel*, *lane 6*), as predicted by the results obtained in yeast (see Fig. 1*B*). However, this NEMO mutant still interacted with transfected IKKβ (Fig. 4*C*, *top panel*, *lane 3*). Moreover, NEMOΔTANK also interacted with CIKS, another NEMO/

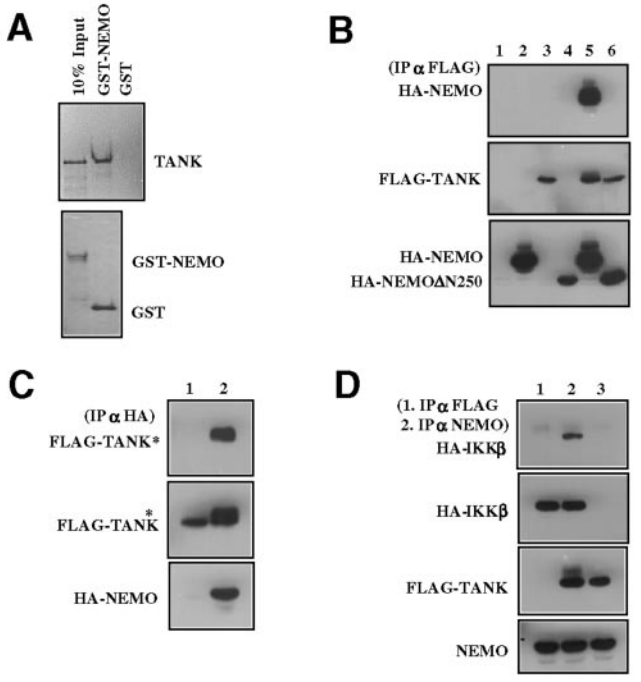

FIG. 2. **TANK interacts with NEMO/IKKγ and the IKK complex.** *A*, *in vitro* protein-protein interaction between HA-TANK and GST-NEMO/IKKγ. *Top panel*, *lane 1*, 0.5 μl of *in vitro* translated HA-TANK; *lane 2* and *3*, 5 μl of *in vitro* translated HA-TANK was used in "pull-down" experiments with GST-NEMO/IKKγ or wild type GST (negative control), respectively. *Bottom panel*, GST-NEMO/IKKγ (*lane 1*) and wild type GST (*lane 2*) visualized on a polyacrylamide gel stained by Coomassie Blue. *B* and *C*, coimmunoprecipitation of FLAG-TANK and HA-NEMO/IKKγ in mammalian cells. 293 cells were transfected with the indicated expression vectors and lysed in 0.5% Triton lysis buffer. Total extracts were immunoprecipitated with anti-FLAG (*B*) or anti-HA (*C*) antibodies, followed by Western analysis with anti-HA or anti-FLAG antibodies (*upper panels*) (*B* and *C*, respectively). The presence of FLAG-TANK, HA-NEMO/IKKγ, and HA-ΔN250 NEMO/IKKγ in the extracts is demonstrated with Western analyses in the *middle* and *lower panels*, respectively. See "Results" for FLAG-TANK*. *D*, evidence for a ternary complex of FLAG-TANK, HA-IKKβ, and endogenous NEMO/IKKγ. 293 cells were transfected with HA-IKKβ (*lane 1*) or FLAG-TANK (*lane 3*) or both (*lane 2*). Cells were harvested 24 h later, and extracts were subjected to an anti-FLAG immunoprecipitation. After extensive washes, the FLAG immunoprecipitates were released from the beads by incubating them with a FLAG peptide overnight. Then a second immunoprecipitation was performed with anti-NEMO/IKKγ antibodies, followed by an anti-HA Western analysis to detect HA-IKKβ (*top panel*). The *bottom three panels* show Western analyses for HA-IKKβ, FLAG-TANK, and endogenous NEMO. *IP*, immunoprecipitation.

IKKγ-interacting protein (22) (Fig. 4*D*, *top panel*, *lane 3*). NEMO/IKKγ thus interacts with TANK via a domain not required for interaction of NEMO/IKKγ with the IKKs or with CIKS. We then tested the ability of the NEMOΔTANK mutant to restore NF-κB activation in NEMO-deficient Jurkat cells (34) in response to stimulation with P/I. Whereas transfection of wild-type NEMO/IKKγ led to significant P/I-induced κB reporter activity, the NEMOΔTANK mutant was largely unable to transmit this signal (Fig. 4*E*). Although this does not prove that interaction with TANK is critical for the function of NEMO, given that as yet unknown functions of NEMO may have been impaired in this particular mutant, the data are nonetheless consistent with the notion that NEMO/IKKγ normally has to bind to proteins such as TANK to be fully functional.

IKKε and TBK1 Promote the Interaction of TANK with the IKK Complex—Two reports identified murine inducible IκB kinase (35) (human homolog termed IKKε (36)) and TBK1 (also named NF-κB-activating kinase (28) and T2K (37)) as two TANK-interacting kinases (27, 35) capable of activating NF-κB in transfection experiments. Inducible IκB kinase and TBK1 were shown to interact with the N-terminal half of TANK and to cause TANK phosphorylation in cotransfection experiments in the C-terminal half (27, 35). Nevertheless, mechanisms for activation of NF-κB by these kinases remained uncertain. We confirmed and extended the published work on the interaction and phosphorylation of TANK with IKKε and TBK1. Both kinases interacted with TANK in the region between amino acids 111 and 169 (just C-terminal to the first of two domains required for interaction with NEMO/IKKγ), and they phosphorylated TANK between amino acids 192 and 247, dependent on the interaction (data not shown).

To investigate whether IKKε may be involved in regulating the ability of TANK to interact with NEMO/IKKγ, 293 cells were transfected with Myc-tagged IKKε (Fig. 5*A*, *lane 1*) or FLAG-TANK (*lane 2*) or combinations of both, using either wild type (*lane 3*) or a K38A kinase-dead (*DN*) mutant of IKKε (*lane 4*). Endogenous NEMO/IKKγ was immunoprecipitated, and the resulting immunoprecipitates were subjected to anti-FLAG and anti-Myc Western analysis (*top two panels*). Exogenously introduced IKKε could not be co-immunoprecipitated with endogenous NEMO/IKKγ (*lane 1*, *second panel from top*), as previously demonstrated (36), whereas FLAG-TANK was detectable only after prolonged exposure (*lane 2*, *top panel*; prolonged exposure not shown). However, co-expression of transfected wild type IKKε strongly promoted the interaction between TANK and NEMO/IKK γ (*lane 3*, *top panel*). Similarly, co-expression of transfected TANK resulted in a readily detectable co-immunoprecipitation of IKKε and NEMO/IKKγ (*lane 3*, *second panel from top*). Interestingly, the K38A IKKε mutant also promoted the interaction between TANK and NEMO/IKKγ, albeit it to a lesser degree, suggesting that the kinase activity of IKKε is not absolutely required for this effect (*lane 4*, *top two panels*).

An analogous experiment was performed in which endogenous IKKα was immunoprecipitated instead of endogenous NEMO/IKKγ (Fig. 5*B*). As expected, little of the exogenously introduced TANK was found in association with IKKα in the absence of transfected IKKε, but the presence of IKKε strongly promoted the interaction of TANK with IKKα, presumably via NEMO/IKKγ (*top panel*, *lanes 2* and *3*, respectively). Again, this effect of IKKε was largely independent of its kinase activity (*lane 4*). Taken together, the results suggest that TANK can link IKKε to the IKK complex and that TANK and IKKε synergize to promote this interaction, largely independent of IKKε kinase activity.

We obtained similar results when TBK1 was tested in these types of experiments. As with IKKε, exogenously introduced TBK1 could be readily found in association with endogenous NEMO/IKKγ, but only in the presence of exogenously introduced TANK (Fig. 5*C*, *lane 3*).

Given that IKKε (and TBK1) promote the association of TANK with the IKKs, although they do not interact with the IKKs by themselves, we tested whether or not a TANK construct lacking the IKKε-interacting domain (FLAG-TANK ΔIKKε; Fig. 6*A*) could still be promoted by IKKε to coimmunoprecipitate with NEMO/IKKγ. We first demonstrated with transfection experiments in 293 cells that such a mutant of TANK indeed failed to interact with IKKε and failed to be phosphorylated by IKKε in an *in vitro* kinase assay but continued to co-immunoprecipitate well with co-transfected NEMO/IKKγ and IKKβ, as predicted (data not shown). Such a mutant allowed us to ask whether IKKε promoted the association of TANK with NEMO/IKKγ by a direct association with TANK or

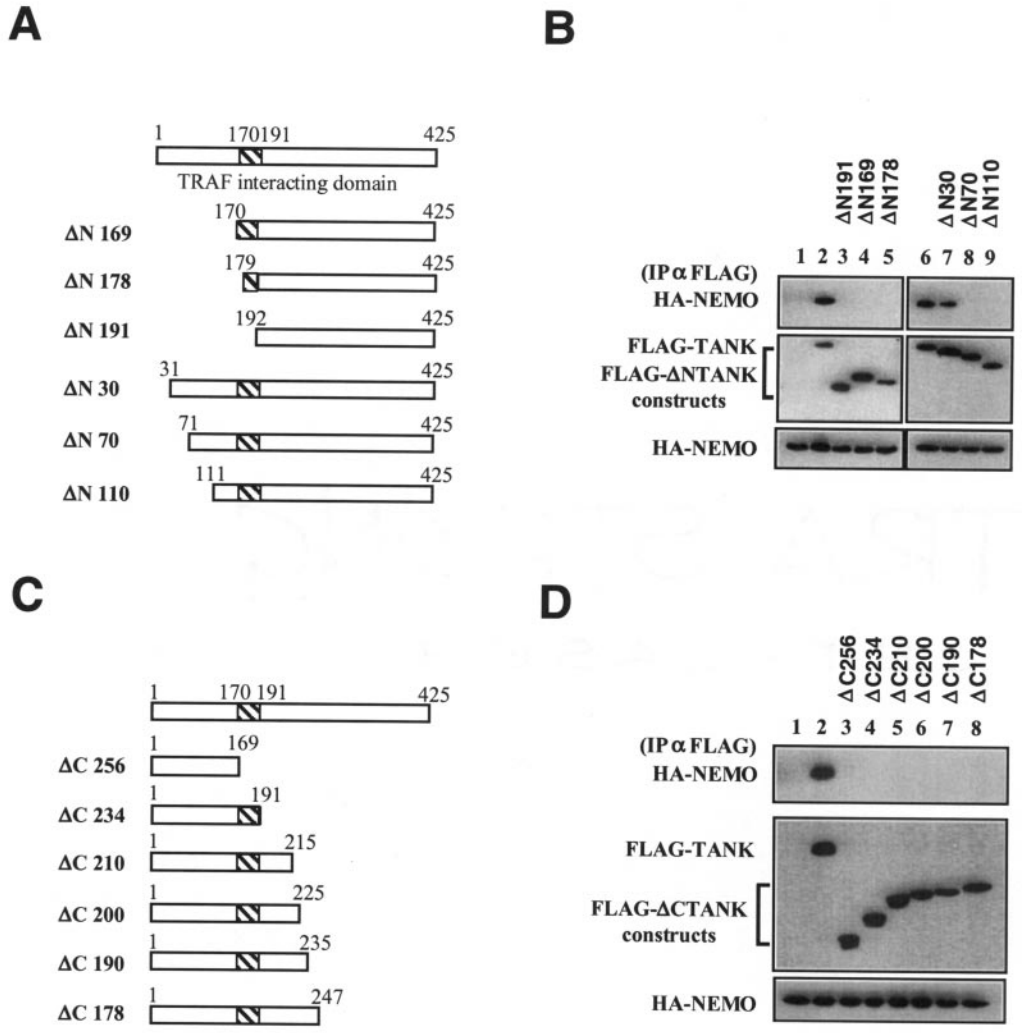

FIG. 3. **Mapping of the NEMO/IKKγ-interacting sites on TANK.** *A* and *C*, schematic illustration of the TANK expression constructs tested for interaction with NEMO/IKKγ. *B* and *D*, coimmunoprecipitations of FLAG-TANK, FLAG-ΔN TANK truncations (*B*), and FLAG-ΔC TANK truncations (*D*) with HA-NEMO/IKKγ. 293 cells were transfected with the indicated expression vector. Total extracts were immunoprecipitated with anti-FLAG antibodies (TANK) followed by Western analyses with anti-HA (NEMO) antibodies (*top panels*). The presence of HA-NEMO/IKKγ and the various TANK truncation mutants are demonstrated with Western analyses in the *middle* and *bottom panels*. *B* (*lanes 2* and *6*) and *D* (*lane 2*) contain full-length FLAG-TANK; the remaining lanes contain TANK mutants as indicated.

whether this might occur indirectly via an effect of IKKε on the IKK complex. As shown in Fig. 6*B* (*lane 5*), the TANK mutant lacking the IKKε binding domain was also no longer promoted by this kinase to interact with endogenous NEMO/IKKγ, whereas wild-type TANK was, regardless of whether IKKε was wild-type or kinase-inactive (Fig. 6*B*, *lanes 2* and *3*, respectively). These results suggest that the direct association of IKKε with TANK allows these proteins to cooperatively interact with NEMO/IKKγ. It is possible, for example, that binding of IKKε changes the conformation of TANK such that it more readily interacts with the IKK complex.

The results also suggest that TANK might be part of a ternary complex with both IKKε and NEMO/IKK γ (and thus the IKK complex). To test such a hypothesis directly, we transfected 293 cells with FLAG-tagged TANK and either wild-type (*WT*) or K38A mutant (*DN*) Myc-tagged IKKε (Fig. 7*A*, *lanes 2* and *3*, respectively). An anti-FLAG immunoprecipitation was carried out, followed by incubation with a FLAG peptide to elute the immunoprecipitated material so that it could be re-immunoprecipitated with antibodies to endogenous NEMO/IKKγ. These final immunoprecipitates were subjected to an anti-Myc Western analysis. In such experiments, we detected both WT IKKε and the K38A (*DN*) mutant (*upper panel*, *lanes 2* and *3*, respectively), indicative of the existence of a ternary complex that includes TANK, IKKε, and endogenous NEMO/IKKγ. The same results were obtained in a similar experiment in which endogenous IKKα was immunoprecipitated instead of endogenous NEMO/IKKγ (Fig. 7*B*). These experiments suggest that ectopically expressed IKKε can be part of a ternary complex with TANK and the IKK complex.

Endogenous TANK Associates with Endogenous IKK, Independent of P/I—IKKε has been described as part of a PMA-inducible IKK-like complex that contains an unknown IKK-like kinase activity (36). We therefore investigated whether stimulation of 293 cells with P/I could modulate the ability of TANK to interact with IKKε or with IKKα, a representative component of the IKK complex (Fig. 8). 293 cells were either left unstimulated or were treated from 15 min to 8 h with P/I prior to harvest, and total cell extracts were subjected to an anti-TANK immunoprecipitation, followed by an anti-IKKε or anti-IKKα Western analysis. As expected, we detected a strong coimmunoprecipitation between endogenous TANK and endogenous IKKε, but this association was not modulated by the P/I treatment (*second panel from top*, *lanes 2–7*). We also observed a much weaker but very reproducible association of endogenous TANK with endogenous IKKα (*top*

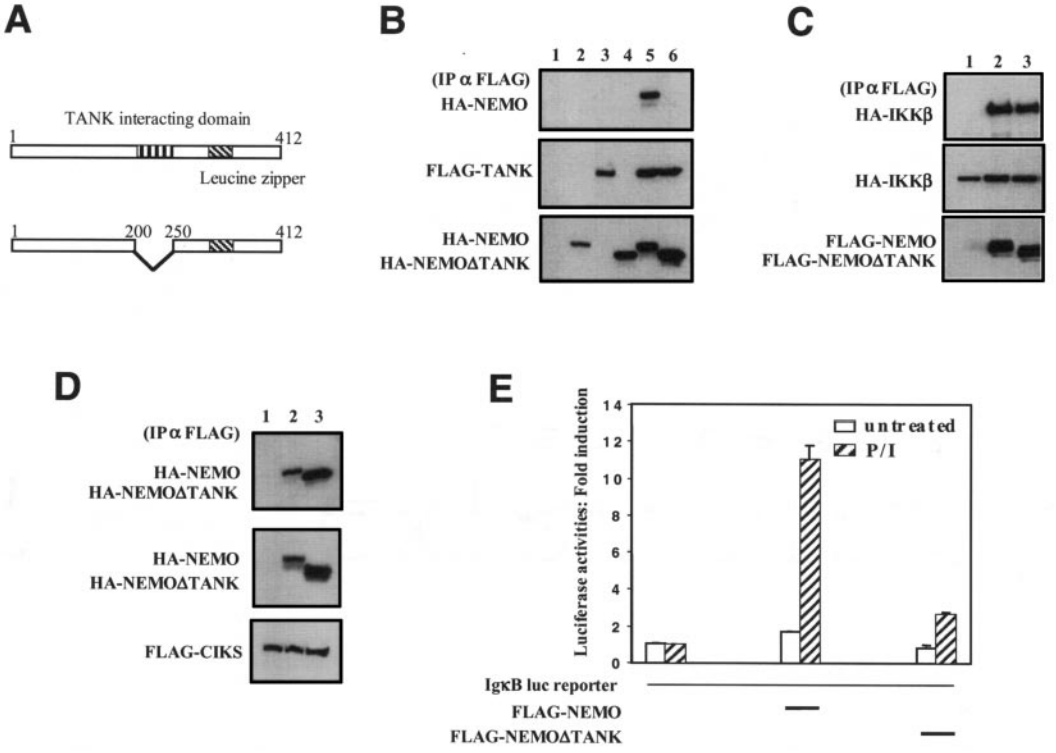

FIG. 4. **NEMO/IKKγ lacking the TANK-interacting site does not fully recapitulate NF-κB activation in NEMO-deficient Jurkat cells.** *A*, schematic representation of the full-length NEMO/IKKγ and NEMOΔTANK constructs. *B*, NEMOΔTANK does not interact with TANK. 293 cells were transfected with the indicated plasmids and lysed in 0.5% Triton lysis buffer. Total extracts were immunoprecipitated with anti-FLAG antibodies (TANK), followed by Western analysis with anti-HA (NEMO) (*top panel*). The presence of FLAG-TANK or of HA-NEMO/IKKγ or HA-NEMOΔTANK in the extracts is shown in the *middle* or *lower panels*, respectively. *IP*, immunoprecipitation. *C* and *D*, NEMO/IKKγ interacts with IKKβ (*C*), CIKS (*D*), and TANK through distinct domains. 293 cells were transfected with the indicated expression vectors and lysed. Total extracts were immunoprecipitated with anti-FLAG antibodies (NEMO (*C*) or CIKS (*D*)), followed by Western analysis with anti-HA (IKKβ (*C*) or NEMO and NEMOΔTANK (*D*)) (*top panels*). The presence of HA-IKKβ or of FLAG-NEMO/IKKγ or NEMOΔTANK (*C*) and of FLAG-CIKS or of HA-NEMO/IKKγ or NEMOΔTANK (*D*) in the extracts is shown with Western analysis in the *middle* and *bottom panels*. *E*, reconstitution of NEMO-deficient Jurkat cells with FLAG-NEMO/IKKγ or FLAG-NEMOΔTANK: NF-κB reporter activity in response to P/I treatment. Shown is the -fold induction of luciferase activity over the basal activity observed with 5 μg of the Ig-κB reporter plasmid alone. Shown are the results of a representative experiment performed in triplicate, after normalization with β-galactosidase activities (mean values ± S.D.). Similar results were obtained in two additional independent experiments.

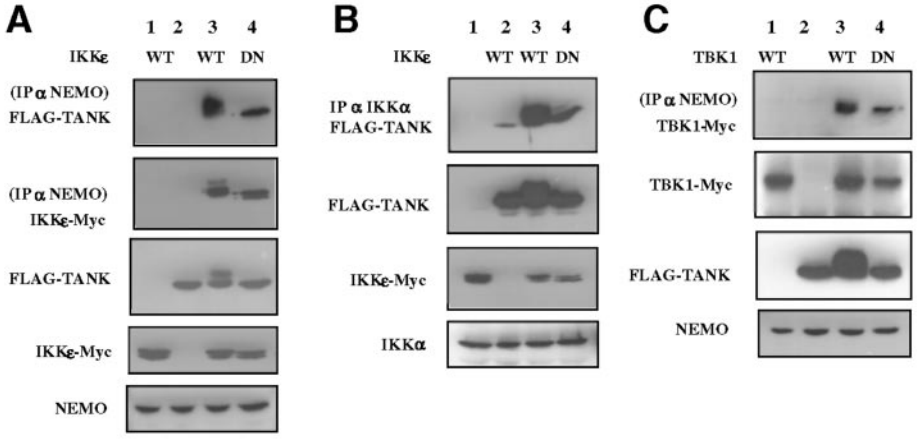

FIG. 5. **IKKε and TBK1 promote the interaction of TANK with the IKK complex.** 293 cells were transfected with FLAG-TANK (*lanes 2*) or with wild type IKKε (*WT*) (*A* and *B*; *lanes 1*) or with wild-type TBK1 (*C*; *lane 1*) or with both FLAG-TANK and wild type IKKε or K38A (*DN*) dominant negative version of IKKε (*A* and *B*; *lanes 3* and *4*, respectively) or with both FLAG-TANK and wild-type or DN TBK1 (*C*; *lanes 3* and *4*, respectively). Total cell extracts were immunoprecipitated with antibodies to endogenous NEMO (*A* and *C*) or antibodies to endogenous IKKα (*B*). TANK (*A* and *B*; *top panels*) and IKKε kinases (*A* and *C*; *second* or *top panel*), respectively, were then detected in these immunoprecipitates by Western analyses with anti-FLAG or anti-Myc antibodies, as shown (in *A*, *second panel from top*, as stripped and reprobed to yield *top panel*). Total cell extracts were also subjected to Western analyses with anti-NEMO/IKKγ, anti-FLAG, anti-Myc, and anti-IKKα antibodies, as indicated (*bottom three panels*).

panel; overnight exposure; IKKε was detected within minutes). Again, the association of TANK with the IKK complex (as demonstrated for IKKα) was not modulated by the P/I stimulation (*top panel*, *lanes 2–7*). These results suggest that P/I treatment, which was hypothesized to activate IKKε has apparently no effect on the ability of TANK to associate with

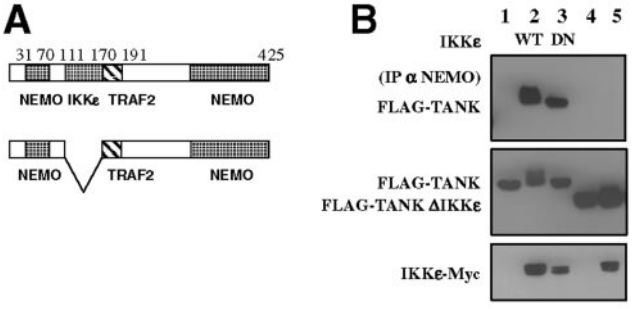

FIG. 6. **IKKε-promoted interaction of TANK with NEMO/IKKγ depends on the ability of TANK to interact with IKKε.** *A*, schematic representation of both the wild type TANK protein and the TANK construct wherein the IKKε interacting domain has been specifically deleted (TANK ΔIKKε). *B*, IKKε does not promote the interaction of TANK ΔIKKε with endogenous NEMO/IKKγ. 293 cells were transfected with FLAG-TANK (*lane 1*) or FLAG-TANK ΔIKKε (*lane 4*) or with FLAG-TANK and Myc-tagged IKKε WT or K38A (*lanes 2* and *3*, respectively) or with FLAG-TANK ΔIKKε and Myc-tagged IKKε; *lane 5*). An anti-NEMO/IKKγ immunoprecipitation was performed followed by Western analysis with anti-FLAG antibodies (*top panel*). The anti-FLAG and anti-Myc Western analyses are shown in the *bottom two panels*.

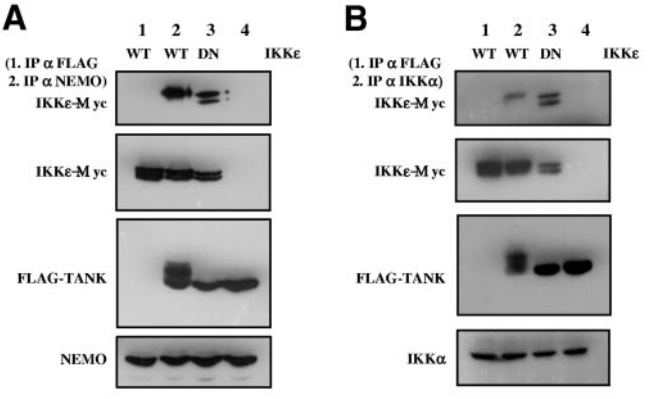

FIG. 7. **Evidence for a ternary complex of transfected TANK, wild type IKKε or K38A mutant IKKε and endogenous NEMO/IKKγ (*A*) or endogenous IKK-α (*B*).** *A* and *B*, 293 cells were transfected with either Myc-tagged IKKε (*lane 1*) or FLAG-TANK (*lane 4*) or with both FLAG-TANK and Myc-tagged IKKε wild type (*lane 2*) or K38A (*DN*) (*lane 3*). Total lysates were immunoprecipitated with anti-FLAG antibodies, and after elution the material was immunoprecipitated with anti-NEMO or anti-IKKα antibodies. Western analyses on final immunoprecipitates were performed with anti-Myc antibodies (*top panels*). Western analyses of total extracts with anti-Myc, anti-FLAG, anti-NEMO/IKKγ, and anti-IKKα antibodies are shown in the *bottom three panels*.

IKKε or to associate with the IKK complex. This is at least consistent with results above that demonstrated that the kinase activity of IKKε is not required for association with the IKK complex via TANK.

DISCUSSION

We have shown here with experiments in yeast, *in vitro* and in transfected cells, that TANK can physically associate with NEMO/IKKγ and thus the IKK complex. Two domains of TANK are required for the interaction with an N-terminal domain of NEMO/IKKγ in mammalian cells. An association of TANK with IKK complexes could also be demonstrated in untransfected cells. Although TANK has been previously implicated in regulation of NF-κB activity, a direct link to NEMO/IKKγ or to the IKK complex has not been reported. This discovery supports the previously suggested notion that NEMO/IKKγ serves an adapter function to link upstream signal mediators with the IKK complex (12). In addition to TANK,

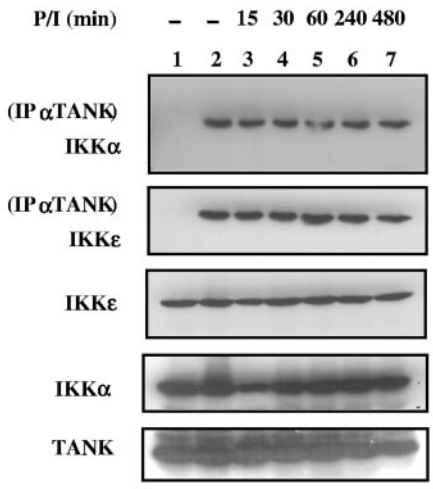

FIG. 8. **Interaction of endogenous TANK with endogenous IKKε and with endogenous IKKα is not modulated by PMA/ionomycin (P/I) treatment.** 293 cells were left unstimulated or treated with P/I from 15 min to 8 h. An anti-TANK immunoprecipitation was performed followed by Western analyses with anti-IKKα (*top panel*, *lanes 2–7*; overnight exposure shown) or anti-IKKε (*second panel from top*, *lanes 2–7*; the anti-IKKα blot was stripped and reprobed with only a few minutes of exposure). An immunoprecipitation using an aliquot of a prebleed rabbit serum was also performed as a negative control (*lane 1*). Western analyses for the relevant proteins in total extracts are shown in the *lower panels*.

other proteins such as the previously identified CIKS (22) (also known as Act1) may also interact with NEMO/IKKγ to link IKKs to select upstream signaling pathways. Some IKKs may be dedicated to specific signaling pathways.

Transfected TANK was reported to negatively affect activation of NF-κB in response to various stimuli (25, 26). The mechanisms for this negative effect remain to be determined, although it was suggested that for some signals, TANK could inhibit by competing with members of the TNF receptor family for binding to TRAF2 (26). Transfected TANK was also reported to positively regulate activation of NF-κB together with low levels of co-transfected TRAF2 (24). This effect was subsequently described to be mediated by the association of TANK with kinases distantly related to IKKα/β, namely TBK1 and IKKε (27, 35). However, what signals these kinases respond to and by what mechanism they may activate NF-κB in concert with TANK has remained unclear. We have shown here that the association of TANK with NEMO/IKKγ and the IKK complex is dramatically increased in the presence of transfected IKKε or TBK1. The physical interaction of IKKε and TBK1 with TANK is sufficient to promote the interaction of TANK with NEMO/IKKγ, whereas their kinase activities are largely dispensable for this effect. IKKε was previously also reported to associate with and regulate an as yet unidentified IKK-like kinase (35). Whereas the present data do not address this issue, they do demonstrate an association of IKKε with the classical IKK kinases, which could of course occur in addition to the association with an unknown IKK-like activity. Our data suggest that TANK may function as an adapter to mediate a direct influence of IKKε and TBK1 on the IKK core complex or on other proteins directly associated with the core IKK complex. We speculate that at least a subset of IKK core complexes exist as part of more loosely assembled, larger signaling complexes that may serve to channel specific activation signals, possibly at special sites within cells. As part of such larger signaling complexes surrounding some IKK cores, TBK1 and IKKε could be in a position not only to directly modulate the IKKα/β kinase activity (27) but conceivably also to regulate

other aspects, such as association of the IKKα/β kinases with their substrates or the phosphorylation of NF-κB proteins.

In attempts to find a functional requirement for the association of TANK with NEMO/IKKγ, we discovered a possible role in mediating activation of NF-κB via P/I. NEMO-deficient Jurkat cells reconstituted with a NEMO mutant lacking the TANK-interacting site (but able to bind CIKS and IKKα/β) are significantly impaired in P/I-induced activation of NF-κB as compared with NEMO-deficient Jurkat cells reconstituted with wild-type NEMO. This suggests that TANK or another protein binding NEMO in the same domain may be required for NEMO to properly channel signals to the IKKs, although alternative explanations cannot be ruled out as yet.

An association of TBK1 or IKKε with the IKK complex could affect NF-κB activity in several ways. In addition to the possibility that TBK1 and IKKε activate the IKKs (28), the association with the IKK complex could also help these kinases modulate other functions, such as the transactivation potential of NF-κB proteins. Such a hypothesis can be derived from T2K-deficient mice. T2K-deficient embryos succumbed to massive apoptosis in the liver, similar to IKKβ and RelA-deficient mice (38–41). In the two latter knockouts, the defect was shown to be due lack of activation of NF-κB in response to TNF, which led to TNF-induced apoptosis, unopposed by the normally protective effects of NF-κB. In T2K-deficient embryonic cells, however, inflammatory cytokine-induced liberation of NF-κB from their IκB inhibitors was shown to be largely intact, and some NF-κB target genes were induced, whereas others were not. Therefore, it was speculated that promoter-specific transactivation functions of liberated NF-κB proteins might be targeted by TBK1. Given the ready access TBK1 and IKKε could have to NF-κB dimers via their association with TANK and the IKK complex (as shown here), these two kinases could well be in position to modulate transactivation functions of NF-κB proteins such as RelA.

In summary, our data provide direct evidence that at least some IKK core complexes can be linked to TANK or other potentially similarly acting proteins. TANK may function as an adapter for the IKKε and TBK1 kinases. These kinases could be liberated and activated by as yet unknown signals so that they may, together with TANK, synergistically engage the IKKs to form a ternary complex. As part of such a hypothesized larger IKK complexes, IKKε and TBK1 could directly modulate activities of the IKK complex. TANK and CIKS may belong to a larger family of adaptors dedicated to link specific signaling pathways to IKK complexes.

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