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Lipopolysaccharide-mediated Interferon Regulatory Factor Activation Involves TBK1-IKKε-dependent Lys^{63}-linked Polyubiquitination and Phosphorylation of TANK/I-TRAF*

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Type I interferon gene induction relies on IKK-related kinase TBK1 and IKKε-mediated phosphorylations of IRF3/7 through the Toll-like receptor-dependent signaling pathways. The scaffold proteins that assemble these kinase complexes are poorly characterized. We show here that TANK/I-TRAF is required for the TBK1- and IKKε-mediated IRF3/7 phosphorylations through some Toll-like receptor-dependent pathways and is part of a TRAF3-containing complex. Moreover, TANK is dispensable for the early phase of double-stranded RNA-mediated IRF3 phosphorylation. Interestingly, TANK is heavily phosphorylated by TBK1-IKKε upon lipopolysaccharide stimulation and is also subject to lipopolysaccharide- and TBK1-IKKε-mediated Lys^{63}-linked polyubiquitination, a mechanism that does not require TBK1-IKKε kinase activity. Thus, we have identified TANK as a scaffold protein that assembles some but not all IRF3/7-phosphorylating TBK1-IKKε complexes and demonstrated that these kinases possess two functions, namely the phosphorylation of both IRF3/7 and TANK as well as the recruitment of an E3 ligase for Lys^{63}-linked polyubiquitination of their scaffold protein, TANK.

The innate immunity in response to a variety of pathogen-associated molecular patterns is established upon binding of their molecular components on specific receptors and ultimately leads to the transcriptional induction of type I interferon (IFN)[8] genes. Signaling pathways triggered by these viral or bacterial products occur through the Toll-like receptor (TLR) or the cytosolic receptor pathway, and both of them rely on the coordinated activation of transcriptional factors, among which the interferon-regulatory factors (IRFs) are critical for the immune response (1–4).

TLRs are members of the so-called pattern recognition receptor family, are mainly expressed on immune system sentinel cells, and specifically sense a variety of molecules produced by bacteria, viruses, fungi, and protozoa (5, 6). For example, lipopolysaccharide (LPS) of Gram-negative bacteria binds TLR4, triggering two distinct signaling pathways, namely the Myd88-dependent and TRIF-dependent pathways. The Myd88-dependent pathway relies on the scaffold proteins TAB2, TAB3, and TRAF6 and ultimately leads to TAK1- and IKK-mediated NF-κB activation and subsequent induction of proinflammatory genes (7, 8). The TRIF-dependent pathway targets IRF3/7 for phosphorylation through a TBK1-IKKε-dependent mechanism, a critical step for type I IFN induction (2). More recently, a TRAF3- and TBK1-IKKε-dependent pathway has also been characterized and appears to be required for the induction of the type I interferons and the anti-inflammatory cytokine IL-10 (9, 10). How TBK1 and IKKε are assembled into functional IRF3/7-phosphorylating complexes is poorly understood. To date, NAP1 is the only NAK/TBK1-interacting scaffold protein identified in the dsRNA-mediated TLR3-dependent, Myd88-independent pathway (11), but it is not known whether NAP1 is involved in other TLR-dependent pathways or whether other scaffold proteins are required.

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[8] The abbreviations used are: HA, hemagglutinin; IFN, interferon; IKK, IκB kinase; IRF, interferon-regulatory factor; ISRE, interferon-stimulated response element; LPS, lipopolysaccharide; Myd88, myeloid differentiation primary response gene 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; TANK, TRAF family member-associated NF-κB activator; TBK1, TANK-binding kinase-1; TNFα, tumor necrosis factor α; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor; TRIF, Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β-mediated transcription factor activation; GST, glutathione *S*-transferase; Ub, ubiquitin; shRNA, short hairpin RNA; dsRNA, double-stranded RNA; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

TANK (also known as I-TRAF) was initially described as a TRAF2/3-interacting molecule that positively regulates NF-κB (12) through an interaction with IKKε (also called IKK-I) (13, 14) and TBK1 (15). TANK/I-TRAF was also characterized as a signaling molecule that negatively regulates NF-κB through its C-terminal domain, but the underlying mechanism remains unclear (16, 17). A role for TANK in NF-κB signaling was further supported by the association of TANK with NEMO/IKKγ, a subunit of the IKK complex (18). This provides a link by which the TANK-interacting TBK1-IKKε kinases are connected to the IKK complex for subsequent phosphorylation of NF-κB proteins, such as p65 or c-Rel (19, 20). Thus, it is believed that TANK acts as a scaffold protein (14, 18). Since the TANK-binding kinases TBK1-IKKε are essential for IRF3/7 activation (21–23), this suggested that TANK may be involved in this and possibly other signaling pathways.

Whereas NF-κB activation through multiple signaling pathways relies on sequentially activated kinases, which ultimately target the inhibitory IκB proteins for phosphorylation and subsequent degradative Lys^{48}-linked polyubiquitination (24, 25), evidence is accumulating that Lys^{63}-linked, nondegradative polyubiquitination of critical scaffold proteins is also essential for IKK and subsequent NF-κB activations (26, 27). Apart from TRAF6 (28), NEMO/IKKγ is the best known example of an NF-κB-activating scaffold protein subject to Lys^{63}-linked polyubiquitination, and several signaling pathways specifically target distinct lysine residues of NEMO/IKKγ (27, 29). This NEMO/IKKγ post-translational modification occurs upon TNFα stimulation and appears to require the E3 ligase c-IAP-1 (30, 31). It also occurs upon T cell receptor signaling, where it relies on MALT-1, a Bcl10-interacting protein potentially acting as an E3 ligase (32). Polyubiquitination of NEMO/IKKγ also occurs through a Nod2-dependent pathway, but the E3 ligase in that signaling cascade remains to be identified (33). This nondegradative polyubiquitination typically requires the E2 protein Ubc13, and although the essential role of this protein in NF-κB activation *in vivo* has recently been questioned, it appears that other pathways, such as those leading to MAPK activation, also involve Lys^{63}-linked and Ubc13-dependent polyubiquitination (34). Like NF-κB activation, the TLR-dependent pathways leading to IRF3/7 activation also rely on sequentially activated kinases, but the extent to which nondegradative Lys^{63}-linked polyubiquitination is required for these signaling pathways is unknown.

To identify scaffold proteins in the TLR- and TBK1-IKKε-dependent pathways and concomitantly learn more about the biological functions of TANK, we searched for TANK-interacting proteins in a yeast two-hybrid screen. TANK was found associated with IRF7 and connects TBK1-IKKε to this protein for subsequent phosphorylation in the TLR- and Myd88-dependent pathways in macrophages. Moreover, we show that TANK is phosphorylated and also subject to Lys^{63}-linked polyubiquitination, both events requiring TBK1-IKKε. Whereas LPS-mediated TANK phosphorylation requires TBK1-IKKε kinase domains, the Lys^{63}-linked TANK polyubiquitination does not require these domains but is TRAF3-dependent. Thus, our results identify TANK/I-TRAF as a signaling molecule positively regulating transcription of type I interferons through some TLR-dependent pathways. We also provide evidence for TBK1-IKKε acting both as IRF3/7 phosphorylating kinases and also as molecules required for the Lys^{63}-linked polyubiquitination of their scaffold protein TANK.

EXPERIMENTAL PROCEDURES

Cell Culture, Biological Reagents, and Mice—Human embryonic kidney 293 and HeLa cells were maintained as described (35, 36), whereas RAW 264.7 macrophages were maintained in Dulbecco's modified Eagle's medium supplemented with 5% low endotoxin fetal bovine serum, glutamine, and antibiotics, respectively. CD14-stably expressing THP1 cells, a gift from Dr. P. Tobias (The Scripps Research Institute, La Jolla, CA) were cultured in RPMI supplemented with fetal bovine serum, glutamine, antibiotics, and G418.

LPS (0111:B4), mouse TNFα, poly(I:C), and CpG DNA (ODN 1826) were purchased from Sigma, Roche Applied Science, Amersham Biosciences, and Invivogen (San Diego, CA), respectively, whereas staurosporin and female BALB/c and C57BL/6 mice were maintained and bred in the laboratory of Parasitology (University of Brussels, Belgium). For isolation of peritoneal macrophages from these mice, sterile inflammation was induced by intraperitoneal injection of thioglycollate 3% in phosphate-buffered saline. Inflammatory peritoneal exudate cells were harvested 48 h after injection using 10 ml of ice-cold and sterile phosphate-buffered saline. Peritoneal macrophages were selected by adherence on cell dishes and were cultured in RPMI supplemented with 5% low endotoxin fetal bovine serum and antibiotics.

Polyclonal anti-human TANK rabbit antibodies were previously described (18). These purified antibodies were used to detect endogenous polyubiquitinated forms of TANK (see below). Anti-Myc, -TRAF3, and -IκBα antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) as were anti-HA beads. Anti-FLAG antibodies and beads were purchased from Sigma. Monoclonal anti-IKKε and -TBK1 antibodies were from Imgenex (San Diego, CA), whereas polyclonal anti-TBK1 antibody was from Cell Signaling (Danvers, MA). The polyclonal and the monoclonal anti-ubiquitin antibodies were purchased from BIOMOL International (Exeter, UK) and Santa Cruz Biotechnology, respectively. The recombinant TBK1 kinase used to assess TANK and IRF3 phosphorylations *in vitro* was from Invitrogen.

Human FLAG-TANK and truncation mutants of TANK were previously described, as were FLAG-TANK ΔIKKε, FLAG-TANKΔZnF, FLAG-IKKε, and the wild type and kinase-dead IKKε-Myc constructs (18, 37). The ISRE reporter plasmid was kindly provided by Dr. R. Beyaert (Department for Molecular Biomedical Research, Unit of Molecular Signal Transduction in Inflammation, VIB-Ghent University, Belgium). The GST-IRF3/7 and -TANK constructs were subcloned by PCR-amplifying the C-terminal portion of IRF3/7 (22) and the full-length TANK coding sequence into the pGex-6P3 (Amersham Biosciences). The corresponding purified fusion proteins were used as substrates for the kinase assays (see below).

The pCW7 Myc-tagged wild type and the pCW8 K48R ubiquitin constructs were a gift from R. Kopito (Department of Biological Sciences, Stanford University), whereas both the Myc-tagged K63R and the K48R/K63R ubiquitin expression constructs were generated by mutagenesis according to standard protocols, using primers whose sequences are available upon request. The previously described HA-tagged ubiquitin as well as the HA-Ub (K0) were provided by Dr. Yarden (Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israël) (38).

Yeast Two-hybrid Screening—The cDNA encoding TANK (amino acids 306–425) was cloned in frame into the GAL4 DNA-binding vector pGADT7 (Clontech). This plasmid was used as bait in a two-hybrid screen of a human HeLa cDNA library in *Saccharomyces cerevisiae* Y187, according to the Matchmaker Two-Hybrid System II Protocol (Clontech). Positive yeast clones were selected for their ability to grow in the absence of histidine, leucine, and tryptophan. Colonies were subsequently tested for β-galactosidase activity, and DNA sequences from positive clones were identified by sequencing.

Immunoprecipitations and Kinase Assays—For immunoprecipitations involving overexpressed proteins, 293 cells (3×10^6) were transfected via FUGENE 6 (Roche Applied Science) with expression vectors as indicated in the figures. 24 h after transfection, cells were washed with phosphate-buffered saline and lysed in 0.5% Triton lysis buffer. Ectopically expressed FLAG- or Myc-tagged proteins were immunoprecipitated by using anti-FLAG or -Myc antibodies bound to agarose beads for 2 h at 4 °C. For anti-TANK immunoprecipitations, cell lysates were incubated with the polyclonal anti-TANK antibody for 2 h followed by an overnight incubation with protein A-agarose. All of the immunoprecipitates were then washed five times with 0.5% Triton lysis buffer and subjected to SDS-PAGE for subsequent Western blot analyses.

To assess IRF3/7 phosphorylation, anti-TANK or -TBK1 immunoprecipitates were used in immune complex kinase assays using a purified GST-IRF3/7 as substrate, as described (36). To assess TANK or IRF3 phosphorylation *in vitro*, the corresponding purified GST fusion proteins were incubated with a recombinant TBK1 kinase, as previously described (36).

In Vivo Ubiquitin Conjugation Assays—293 cells were transfected with Myc- or HA-ubiquitin and either FLAG-TANK or FLAG-TANK mutants together with the indicated expression vectors, according to the protocol described above. 24 h after transfection, cells were lysed, and total cell extracts were subjected to anti-FLAG immunoprecipitations using the anti-FLAG beads, as described above. The ubiquitin-conjugated TANK proteins were subsequently detected by performing anti-Myc or -HA Western analyses.

For detection of endogenous polyubiquitinated forms of TANK, cell extracts were lysed as described (30) and subsequently incubated overnight with the purified anti-TANK antibody followed by a 2-h incubation with protein A-agarose. Immunoprecipitates were subsequently subjected to anti-Ub western analyses.

RNA Interference and Luciferase Assays—For RNA interference, decreased Ubc13 expression was obtained by transfecting a SMART POOL of Ubc13 (Dharmacon, CO) using the oligofectamine reagent according to the protocol provided by the manufacturer (Invitrogen).

For generation of the shRNA constructs targeting either green fluorescent protein (control) or the TANK transcript, inserts were cloned into the pLL3.7 lentivirus according to the protocol kindly provided by Dr. L. van Parijs (MIT, Boston, MA) (39). Details are available upon request. For TRAF3 depletion in human macrophages, THP1/CD14 cells were transfected with either the MISSION shRNA lentiviral construct targeting the TRAF3 transcript or the MISSION nontarget shRNA control vector, as described by the manufacturer (Sigma).

For luciferase assays, 293 cells (4×10^5 cells/well) were seeded in 6-well (35-mm) plates. After 12 h, cells were transfected as described above with 1 μg of the reporter plasmid and with expression plasmids as indicated. The total amount of transfected DNA was kept constant by adding empty expression vector DNA as needed. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined by the luciferase assay system (Roche Applied Science). A pGL4.74 plasmid (Promega, Madison, WI) was used to normalize for transfection efficiencies.

RESULTS

TANK Associates with IRF3 and IRF7 through its C-terminal Domain—In order to identify TANK-dependent signaling pathways other that those leading to NF-κB activation, we searched for proteins that physically interact with this scaffold protein by means of a yeast two-hybrid screen. The C-terminal domain of TANK (amino acids 306–425) fused to the DNA binding domain of the GAL4 transcription factor (Fig. 1*A*, *left*) was used to screen a human HeLa cDNA library that expresses the encoded proteins as fusions with the GAL4 transactivation domain. Among clones that were scored positive for interaction with the bait, one encoded the C-terminal domain and regulatory region of IRF7 (Fig. 1*A*, *right*). The interaction between IRF7 and TANK was confirmed by co-immunoprecipitation. Ectopically expressed Myc-tagged IRF7 co-immunoprecipitated with FLAG-TANK in 293 cells (Fig. 1*B*, *top*, *left*, *lane 2*). Myc-tagged IRF3 also bound FLAG-TANK (Fig. 1*B*, *top*, *right*, *lane 2*). To confirm that the C-terminal domain of TANK is required for binding to IRF3/7 in mammalian cells, similar co-immunoprecipitations were performed with extracts from 293 cells transfected with Myc-IRF7 and either FLAG-TANK or various TANK mutants deleted in the C-terminal domain. Wild type TANK bound IRF7, but a TANK mutant lacking the 178 C-terminal amino acids did not (Fig. 1*C*, *top*, *lanes 2* and *6*, respectively). A TANK mutant with two point mutations within its zinc finger motif ("TANKΔZnF"), which disrupt a NEMO-binding domain (37), still associated with IRF7, as did TANKΔC20 and TANKΔC50 (Fig. 1*C*, *top*, *lanes 3–5*). Therefore, TANK binds IRF7 through a C-terminal region that is distinct from the domain required for association with NEMO/IKKγ.

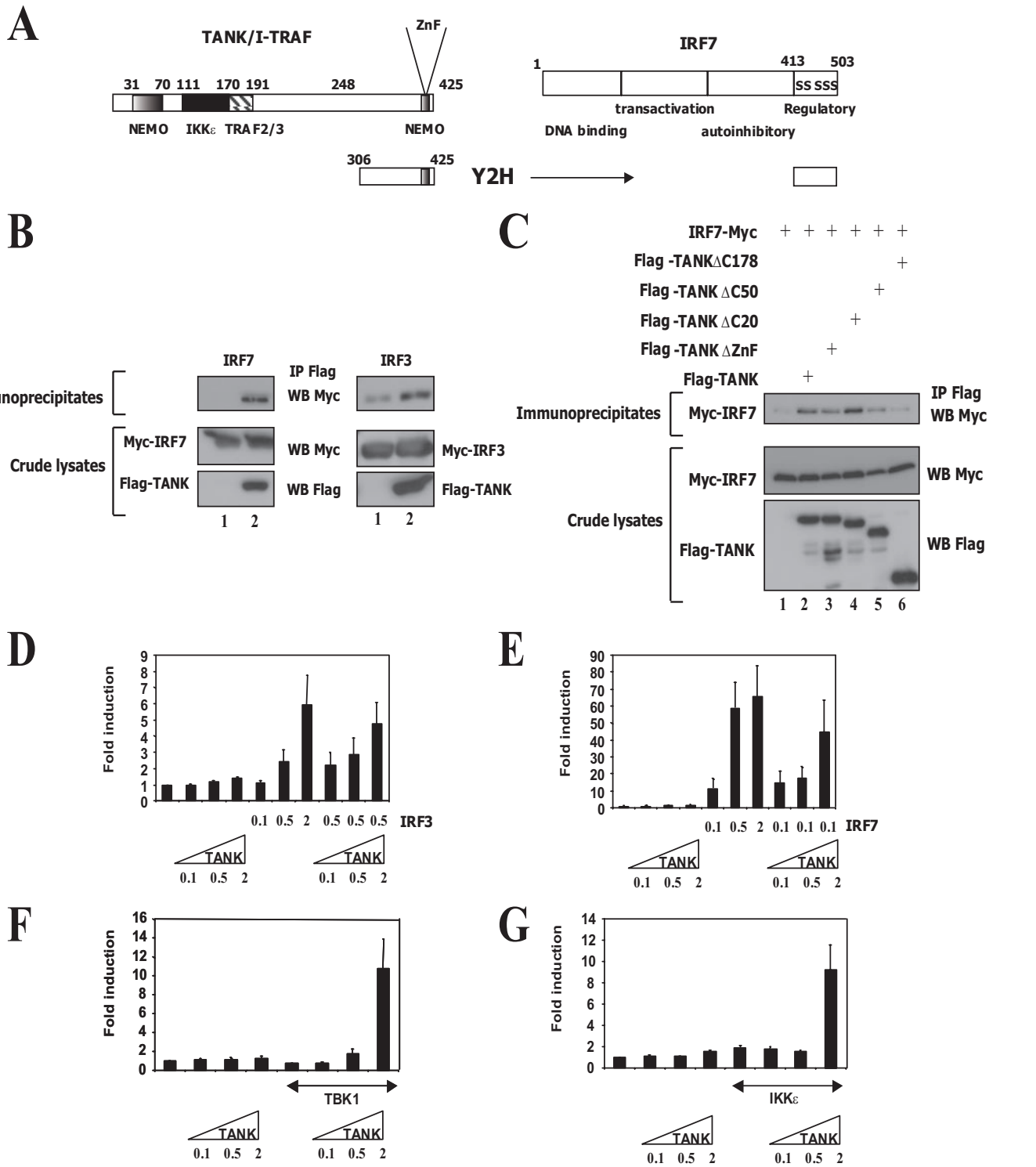
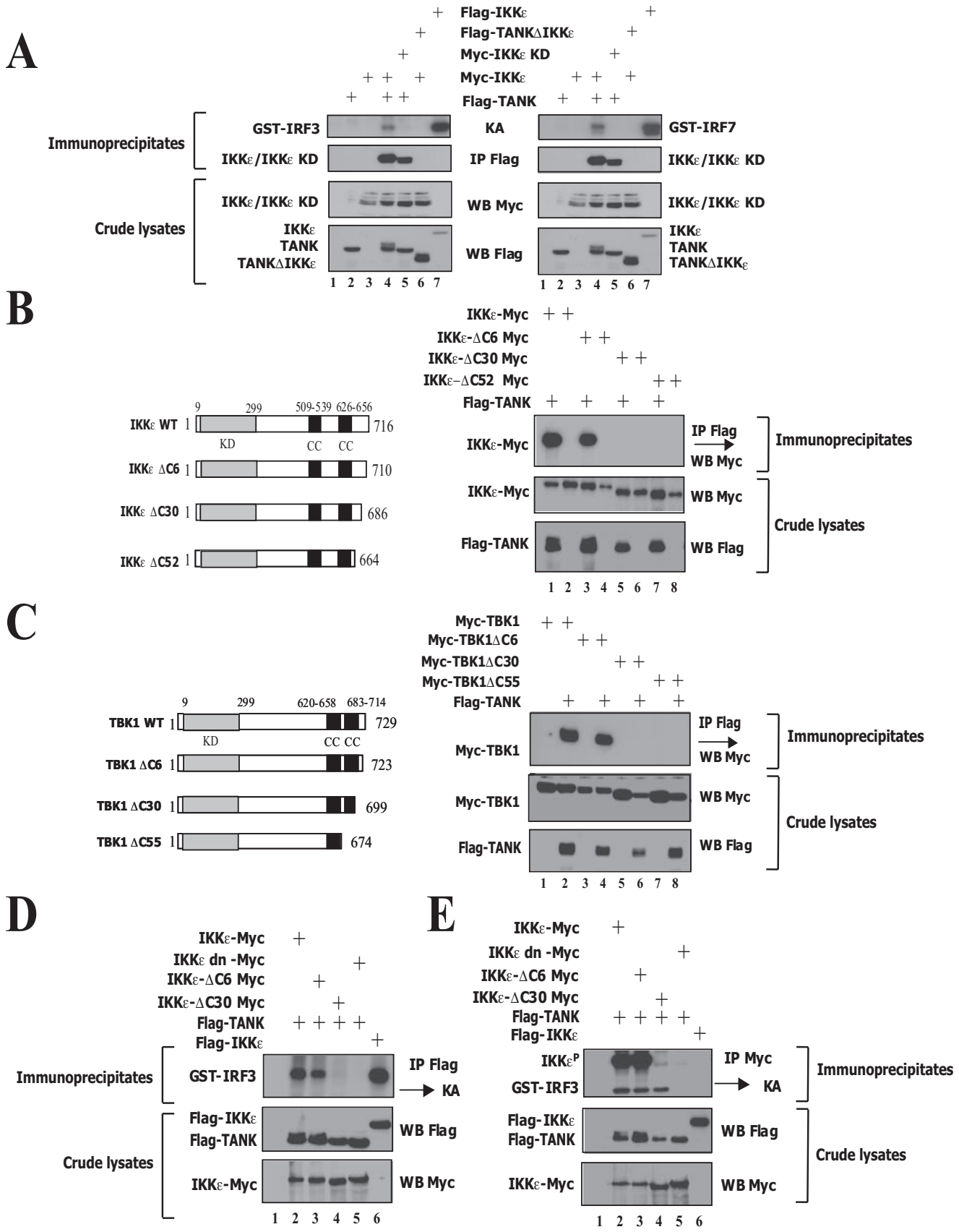


FIGURE 1. **TANK binds IRF7 and -3 and enhances IRF3/7-mediated interferon transcriptional induction.** *A*, *left*, schematic representation of both TANK and the bait used in yeast two-hybrid analyses (*Y2H*). The NEMO-, IKKε-, and TRAF2/3-interacting domains on TANK are depicted. *Right*, schematic representation of full-length IRF7 and the cDNA clone pulled out from the yeast two-hybrid screening. The IRF7 functional domains are illustrated as well. *SS SSS*, serine residues. *B*, ectopically expressed FLAG-TANK binds Myc-tagged IRF3 and -7. *Left*, 293 cells were transfected with Myc-IRF7 or in combination with FLAG-TANK (*lanes 1* and *2*, respectively), and cell lysates were subjected to anti-FLAG immunoprecipitations (*IP*) followed by anti-Myc Western analyses (*WB*) (*top panel*). Cell lysates were subjected to anti-Myc and -FLAG Western analyses as well (*second* and *third panel* from the *top*, respectively). *Right*, identical experiment except that Myc-IRF7 was replaced by Myc-IRF3. *C*, TANK binds IRF7 through its C-terminal domain. 293 cells were transfected with Myc-IRF7 alone (*lane 1*) or with either FLAG-TANK or FLAG-TANK mutants (*lanes 2–6*), as indicated *above* the *panels*. Cell lysates were subjected to anti-FLAG immunoprecipitations followed by anti-Myc Western analyses (*top*). Anti-Myc and -FLAG analyses were carried out on the cell extracts as well (*second* and *third panels* from the *top*). *D–G*, TANK enhances IRF3 (*D*), IRF7 (*E*), TBK1 (*F*), or IKKε (*G*)-mediated transcription of interferon. The figure shows relative luciferase activities observed in 293 cells transfected in duplicate with 0.5 μg of ISRE luciferase reporter plasmid, with or without the indicated expression vectors, as indicated. Values shown (in arbitrary units) represent the means ± S.D. of at least three independent experiments, normalized for *Renilla* luciferase activities of a cotransfected pGL4.74 plasmid.

TANK Enhances IFN Transcription through TBK1 and IKKε-mediated IRF3/7 Activation—Because TBK1-IKKε-mediated IRF3/7 phosphorylation is essential for the transcriptional induction of type I interferon and subsequent development of the innate response, we next determined whether TANK, as a TBK1-IKKε- and IRF3/7-binding protein, is involved in IRF3/7 activation. 293 cells were transfected with the ISRE reporter plasmid, which harbors an IRF3/7-responsive element, and luciferase assays were performed. As expected, increasing amounts of IRF3/7 led to a dose-dependent activation of the ISRE reporter; TANK overexpression alone did not (Fig. 1, *D* and *E*, respectively). The addition of increasing amounts of TANK weakly enhanced IRF3-mediated activation of the ISRE promoter and more strongly induced IRF7 transactivation potential (Fig. 1, *D* and *E*, respectively). TANK also enhanced TBK1- and IKKε-mediated activation of the ISRE promoter (Fig. 1, *F* and *G*). Therefore, these results suggest that TANK positively regulates TBK1- and IKKε-mediated IRF3/7 activation, presumably by connecting both TANK-interacting kinases to their substrate for its subsequent phosphorylation.

TANK Is Part of an IRF3/7-phosphorylating Complex—To explore the hypothesis that TANK connects TBK1-IKKε to their substrates, we addressed IRF3/7 phosphorylations in cells overexpressing IKKε and either wild type TANK or a mutant lacking the TBK1-IKKε-interacting site ("TANKΔIKKε") (18) by kinase assays using purified GST-IRF3 or -7 as substrate. In agreement with previous reports, IRF3 and IRF7 were strongly phosphorylated by an anti-FLAG immunoprecipitate derived from FLAG-IKKε- but not Myc-IKKε-overexpressing cells (Fig. 2*A*, *top*, *lanes 7* and *3*, respectively). Moreover, whereas IRF3/7 phosphorylations were detected by incubating anti-FLAG immunoprecipitates derived from cells expressing both FLAG-TANK and IKKε-Myc (Fig. 2*A*, *top*, *lanes 4*), no IRF3/7 phosphorylation was detected using immunoprecipitates derived from cells overexpressing a kinase-dead version of IKKε or TANKΔIKKε (Fig. 2*A*, *top*, *lanes 5* and *6*, respectively). We conclude, therefore, that TANK connects IKKε to IRF3/7 for subsequent phosphorylation. To further explore the significance of the interaction between TANK and TBK/IKKε for IRF3/7 activation, we first defined the TANK-interacting site on both kinases by co-immunoprecipitations. TBK1 and IKKε harbor a N-terminal kinase domain as well as C-terminal coiled-coil regions (40). C-terminal IKKε/TBK1-deletions were generated, and the resulting Myc-tagged wild type and IKKε/TBK1 mutants were tested for interaction with FLAG-TANK (Fig. 2, *B* and *C*, respectively). IKKε/TBK1 and IKKεΔC6/TBK1ΔC6-Myc associated with FLAG-TANK (Fig. 2, *B*, *top*, *lanes 1* and *3*, and *C*, *top*, *lanes 2* and *4*), but IKKε/TBK1 mutants lacking 30 or 52 (for IKKε) and 30 or 55 (for TBK1) C-terminal amino acids failed to interact with FLAG-TANK (Fig. 2, *B*, *top*, *lanes 5* and *7*, and *C*, *top*, *lanes 6* and *8*). This indicates that IKKε and TBK1 interact with TANK through their C-terminal regions downstream of the coiled-coil domains. We next defined the role of the TANK-interacting domain of IKKε in IRF3 activation by assessing IRF3 phosphorylation in kinase assays using extracts of cells with ectopic wild type or IKKε mutants deleted in the coiled-coil domains ("IKKεΔC6" or "IKKεΔC30") by kinase assays. IRF3 phosphorylation was detected following incubation with anti-FLAG immunoprecipitates derived from cells expressing FLAG-IKKε or both FLAG-TANK and wild type but not kinase-dead IKKε-Myc (Fig. 2*D*, *top*, *lanes 6*, *2*, and *5*, respectively). Also, whereas IRF3 phosphorylation was detected in cells overexpressing FLAG-TANK and IKKεΔC6, (which still interacts with TANK), no IRF3 phosphorylation was detected upon FLAG-TANK and IKKεΔC30 overexpression (Fig. 2*D*, *top panel*, *lanes 3* and *4*, respectively). This result provides further support for the hypothesis that TANK is associated with an IRF3 kinase, most likely IKKε. We next performed a kinase assay with immunoprecipitates of wild type and IKKε mutants rather than TANK. As expected, IRF3 phosphorylation was detected using anti-Myc imunoprecipitates derived from cells overexpressing IKKε-Myc but not the kinase-dead mutant or FLAG-IKKε (Fig. 2*E*, *top*, *lanes 2*, *5*, and *6*, respectively). IRF3 phosphorylation was also detected in extracts of cells overexpressing IKKεΔC6 or IKKεΔC30 (Fig. 2*E*, *top*, *lanes 3* and *4*, respectively). Interestingly, whereas IKKε autophosphorylation was observed upon overexpression of wild type or the IKKεΔC6 mutant, such autophosphorylation was disrupted by deleting the last 30 amino acids of IKKε (Fig. 2*E*, *top*, *lanes 3* and *4*, respectively). These results suggest that the N-terminal IKKε kinase domain is sufficient for IRF3 phosphorylation *in vitro*, whereas the C-terminal coiled coil domain required for TANK interaction is dispensable for IRF3 phosphorylation but required for IKKε autophosphorylation *in vitro*.

TANK Is Involved in the LPS-mediated IRF3/7-activating Pathway—To demonstrate the existence of an endogenous, signal-responsive TANK-containing protein complex that phosphorylates IRF3/7, we first stimulated RAW 264.7 macrophages with LPS, which is known to activate TBK1-IKKε through the TLR4-dependent pathway (23, 41), or with TNFα and subjected the cell lysates to anti-TANK immunoprecipitations followed by an anti-IRF3 Western blot. Interestingly, IRF3 physically associated with TANK upon LPS but not TNFα stimulation, and this interaction was transient, since such a TANK-IRF3 complex was only detectable upon 15 min of stimulation (Fig. 3*A*, *top*, *lane 2*). To further investigate whether such signal-responsive interaction causes IRF3/7 phosphorylations, RAW 264.7 macrophages were again stimulated with LPS or TNFα and subsequently subjected to anti-TANK or -HA (negative control) immunoprecipitation followed by an *in vitro* kinase assay using GST-IRF7 as substrate. LPS-dependent IRF7 phosphorylation was observed again upon 15 min of treatment, which perfectly matches with the kinetics of the TANK-IRF3 interaction, and decreased but persisted even after 4 h of stimulation (Fig. 3*B*, *top*, *lanes 2–7*). Of note, the association of IKKε or TBK1 with TANK was detected in unstimulated cells and was not modulated by LPS as judged by anti-IKKε or -TBK1 Western analysis performed on the anti-TANK immunoprecipitates (Fig. 3*B*, *second* and *third panels* from the *top*, compare *lane 2* with *lane 7*). Moreover, a much weaker IRF7 phosphorylation was detected upon TNFα stimulation (Fig. 3*C*, *top*, *lanes 3* and *4*). A similar experiment was performed using peritoneal macrophages harvested from a thioglycollate-treated mice. This also showed that IRF7 was phosphorylated by a



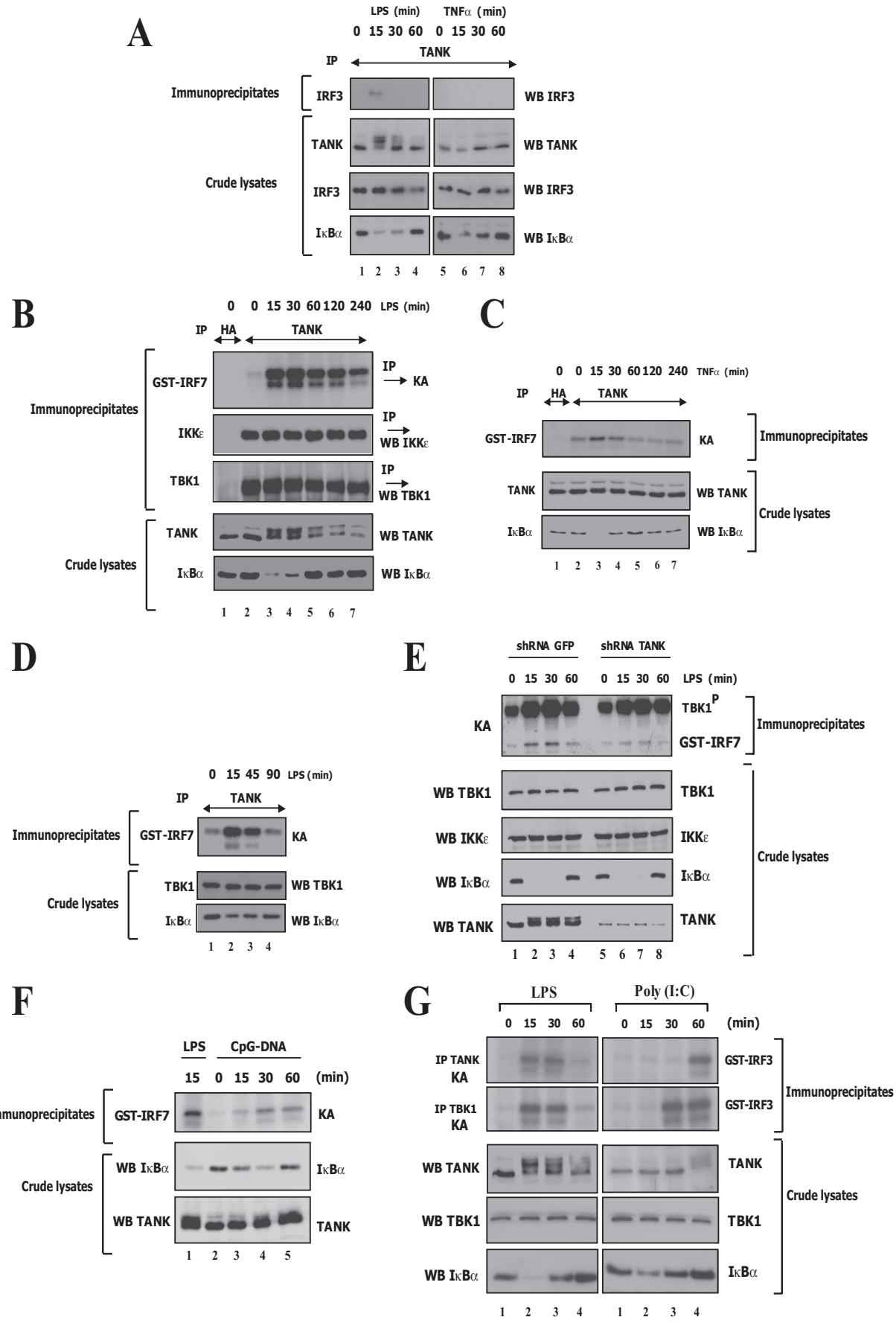
TANK-containing complex in LPS-stimulated cells (Fig. 3*D*, *top*, *lanes 2* and *3*). These data shows that TANK is part of an LPS-inducible IRF3/7-phosphorylating protein complex *in vivo*. Slower migrating forms of TANK due to phosphorylation (see below) were detected upon LPS but not TNFα stimulation (Fig. 3, *A*, *left*, *second panel* from the *top*, *lanes 2* and *3*, and *B*, *second panel* from the *bottom*, *lanes 3–6*). It is likely that TBK1-IKKε are involved in this post-translational modification of TANK, since both of them were previously described as TANK kinases *in vitro* (14, 15). We next infected LPS-responsive human CD14-stably expressing THP1 cells with an shRNA construct targeting either the TANK transcript or green fluorescent protein (negative control) and assessed LPS-mediated IRF7 phosphorylation in those cells. This signaling pathway was impaired upon TANK depletion, as evidenced by IRF7 phosphorylation (Fig. 3*E*, *top*, compare *lanes 1–4* with *lanes 5–8*). Therefore, TANK is required to assemble a functional IRF7-phosphorylating TBK1-IKKε complex in the LPS-mediated signaling pathway.

TANK Assembles TBK1-IKKε in the TLR-, TRAF3-dependent Pathways—Because IRF7 phosphorylation also occurs through the TLR9- and Myd88-dependent pathway upon stimulation with unmethylated DNA (42), we asked whether TANK also connects TBK1-IKKε to IRF7 in this pathway and indeed found that anti-TANK immunoprecipitates from CpG-treated RAW 264.7 cells harbored an IRF7-phosphorylating activity (Fig. 3*F*, *top*, compare *lane 2* with *lanes 3–5*). Therefore, these results suggest that TANK is a scaffold protein that assembles the TBK1-IKKε complex for IRF3/7 phosphorylation in the TLR4- but also the TLR9-dependent signaling pathways. We next stimulated RAW 264.7 cells with poly(I:C), which is known to trigger IRF3 activation through the TLR3 and -TRIF dependent but Myd88-independent pathway and determined whether or not a TANK immune complex can indeed phosphorylate IRF3 through that pathway. As expected, anti-TBK1 immunoprecipitates indeed phosphorylated IRF3 upon LPS and poly(I:C) stimulations (Fig. 3*G*, *second panels* from the *top*). Interestingly, whereas anti-TANK immunoprecipitates caused IRF3 phosphorylation after 15 min of stimulation by LPS, TANK-mediated IRF3 phosphorylation upon poly(I:C) only occurred after 60 min of stimulation (Fig. 3*G*, *top left* and *right panels*, respectively). Therefore, another scaffold protein, potentially NAP1 (see below), may be required for early IRF3 phosphorylation through the TLR3 pathway.

Because TRAF3 has been identified as a critical signaling molecule for type I interferon induction through the TLR-dependent pathways (9, 10), we next investigated whether TANK, TBK1, and TRAF3 are part of a common signaling complex by co-immunoprecipitation studies. 293 cells were transfected with FLAG-TANK with or without TBK1-Myc, and anti-FLAG immunoprecipitations were performed. The immunoprecipitates were released from the beads by incubating them with a FLAG peptide, and the released material was immunoprecipitated with antibodies to endogenous TRAF3, followed by an anti-Myc Western analysis, which revealed the presence of TBK1-Myc (Fig. 4*A*, *lane 3*). Therefore, a ternary complex of TANK, TBK1, and TRAF3 must have been formed in these cells. The hypothesis that these latter proteins are part of a common LPS-inducible signaling complex was further supported by the fact that LPS enhanced association of TRAF3 with TBK1 in stimulated RAW 264.7 cells (Fig. 4*B*, *top*, compare *lane 1* with *lanes 2* and *3*). Thus, our results suggest that TANK connects TBK1-IKKε for IRF3/7 phosphorylation in the TLR-, Myd88-, and TRAF3-dependent pathways.

LPS-mediated TANK Phosphorylation in Macrophages—We observed that LPS stimulation of RAW 264.7 macrophages led to the appearance of slower migrating forms of TANK in Western blot analysis (Fig. 3*A*). These forms were detected after 15 min of stimulation at two LPS concentrations used to treat these cells (Fig. 5*A*, *top*, *lanes 2–7*). Moreover, these slower migrating forms of TANK were also detected in LPS-stimulated but not in TNFα-stimulated THP1 cells, which stably express CD14 (Fig. 5*B*, *top*, compare *lanes 1* with *lanes 2–5*). To test whether the slower migrating species were due to protein phosphorylation, extracts from LPS-stimulated RAW 264.7 cells were incubated with λ-phosphatase and subjected to anti-TANK Western analysis. We observed that the phosphatase treatment led to the disappearance of the slower migrating TANK forms (Fig. 5*C*, compare *lanes 2* and *3*). Additional evidence for TANK phosphorylation was obtained when RAW 264.7 cells were preincubated with staurosporin, a kinase inhibitor, before stimulation with LPS. The LPS-induced TANK phosphorylation was totally abolished upon staurosporin treatment (Fig. 5*D*, compare *lanes 2–4* and *lanes 6–8*). Taken together, our results suggest that TANK is subjected to a massive LPS-mediated phosphorylation. To determine whether TBK1-IKKε are the TANK-phosphorylating kinases, we mapped the domains in TANK required for interaction with and/or phosphorylation by overexpressed IKKε or TBK1. To do so, FLAG-TANK was transiently co-expressed in HeLa cells together with Myc-tagged IKKε or TBK1. Anti-FLAG immunoprecipitations were carried out on the cell extracts, and the resulting immunoprecipitates were subjected to anti-Myc Western blots as well as to *in vitro* kinase assays using immunoprecipitated FLAG-TANK as substrate (Fig. 5*E*). Both overexpressed IKKε and TBK1 kinases interacted with (Fig. 5*E*, *sec-*

FIGURE 2. **TANK is part of an IRF3/7-phosphorylating complex.** *A*, an anti-TANK immunoprecipitate phosphorylates IRF3/7 in transfected cells. 293 cells were transfected with the indicated expression vectors, and cell lysates were subjected to anti-FLAG immunoprecipitations (*IP*). IRF3 and -7 phosphorylations were assessed by performing *in vitro* kinase assays using the anti-FLAG immunoprecipitates and the purified GST-IRF3/7 fusion proteins as substrates (*top panels* on the *left* and on the *right*, respectively). Anti-FLAG/Myc Western analyses (*WB*) were carried out on the cell lysates as well (*bottom panels*). *B* and *C*, IKKε/TBK1 interact with TANK through their C-terminal coiled-coil domains. *Left*, schematic representation of the IKKε (*B*) and TBK1 (*C*) constructs tested for interaction with TANK. The coiled-coil domains (*CC*) are depicted by *black boxes*, whereas the kinase domains (*KD*) are shown by *gray rectangles*. On the *right*, 293 cells were transfected with the indicated expression plasmids, and anti-FLAG immunoprecipitations followed by anti-Myc Western analysis were carried out (*top*). Anti-Myc and -FLAG Western analyses were carried out on the cell lysates as well (*middle* and *bottom*, respectively). *D* and *E*, the C-terminal and TANK-interacting coiled-coil domains of TBK1-IKKε are dispensable for IRF3 phosphorylation *in vitro*. 293 cells were transfected with the indicated expression plasmids, and anti-FLAG (*D*) or -Myc (*E*) immunoprecipitations were carried out. The resulting immunoprecipitates were subjected to *in vitro* kinase assays using the purified fusion protein GST-IRF3 as substrate (*top*). Anti-FLAG and -Myc Western analyses were carried out on the cell lysates as well (*middle* and *bottom*, respectively). *IKKε*^{p}, IKKε autophosphorylation. *WT*, wild type.



ond panels from the *top*, *lanes 3*) and phosphorylated TANK (Fig. 5*E*, *top panels*, *lane 3*). These experiments were repeated using various ΔN and ΔC TANK expression constructs. When co-expressed with IKKε, FLAG-ΔN70, -ΔN110, and -ΔC178 TANK were still phosphorylated, whereas FLAG-ΔC256 and -ΔC234 TANK were not (Fig. 5*E*, *top panel* on the *left*), despite the fact that all of these truncations still interacted with IKKε (Fig. 5*E*, *second panel* from the *top*). FLAG-ΔN169 TANK was not phosphorylated by IKKε, but it also no longer interacted with this kinase (Fig. 5*E*, *top panel* and *second panel* from the *top*, respectively, *lane 6*). These results suggest that TANK interacts with IKKε in the domain lying between amino acids 111 and 169, just C-terminal to the first of two domains required for interaction with NEMO/IKKγ (18) and that TANK is phosphorylated between amino acids 192 and 247. These experiments were repeated with FLAG-tagged TANK and TBK1 expression constructs and led to identical results (Fig. 5*E*, *panels* on the *right*), although the expression levels for TBK1 were somewhat below those for IKKε. As a proof for TANK being a direct substrate of TBK1, we subjected a purified GST-TANK fusion protein or the GST-IRF3 (positive control) to an *in vitro* kinase assay using a recombinant TBK1 kinase. Both IRF3 and TANK were indeed phosphorylated by TBK1 *in vitro* (Fig. 5*F*, on the *right*, *lanes 2* and *4*, respectively). Our results suggest that IKKε and TBK1 behave similarly in terms of their interaction with and phosphorylation of TANK.

TBK1-IKKε but Not IKKβ Triggers TANK Polyubiquitination through a Phospho-independent Pathway—Because evidence is accumulating that polyubiquitination critically regulates signal transduction (43, 44) and because scaffold proteins, such as NEMO/IKKγ, are subjected to this post-translational modification, we investigated whether the LPS-inducible and TANK-phosphorylating kinase IKKε causes TANK polyubiquitination. To address this issue, 293 cells were transfected with FLAG-TANK, HA-Ub, or a mutant where all lysines are mutated, HA-Ub (K0), with or without IKKε-Myc. Anti-HA Western analyses were performed on the anti-FLAG immunoprecipitates to detect polyubiquitin-conjugated TANK adducts. Co-expression of FLAG-TANK and HA-Ub did not lead to TANK polyubiquitination (Fig. 6*A*, *top*, *lane 2*). However, polyubiquitination of TANK was detected upon its co-expression with HA-Ub and IKKε-Myc (Fig. 6*A*, *lane 3*). IKKε-mediated TANK

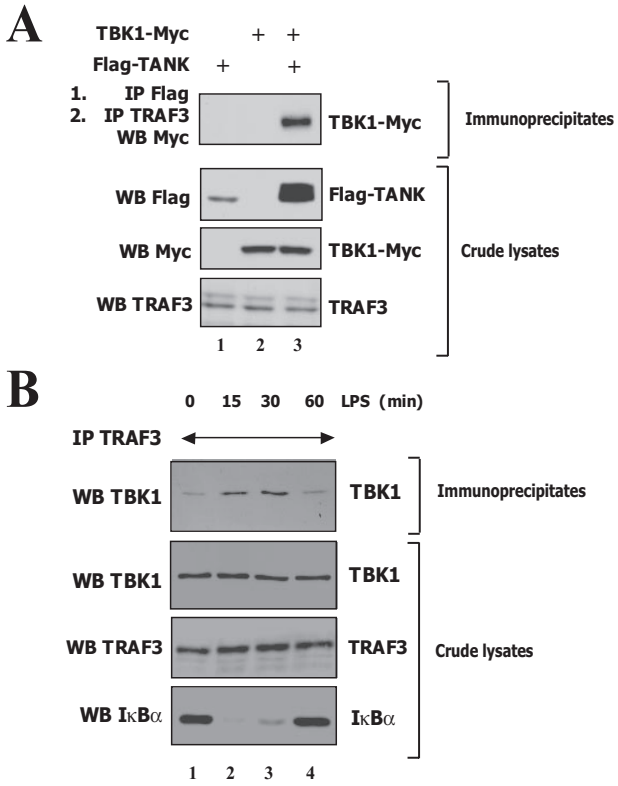
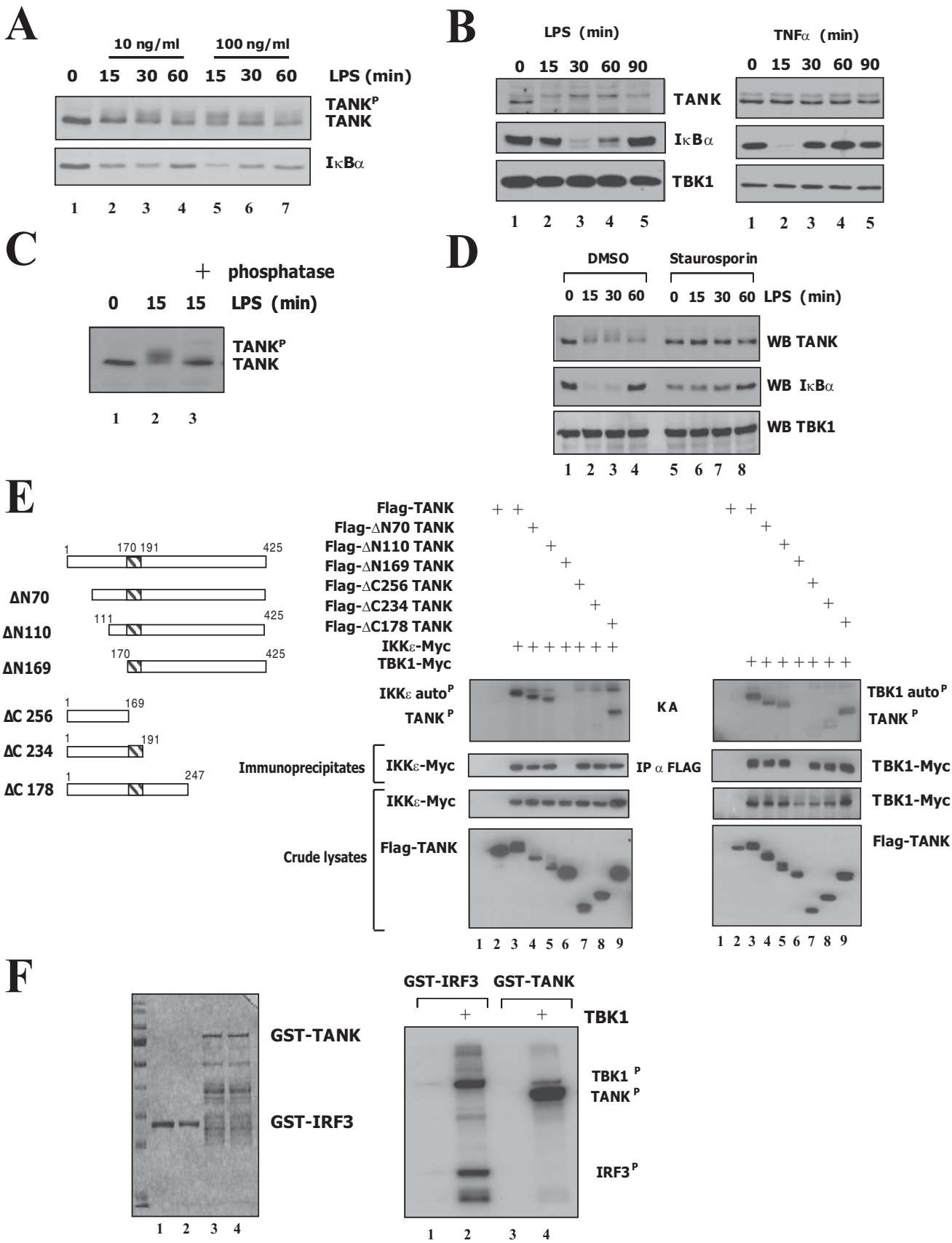


FIGURE 4. **TANK is associated with TRAF3 and TBK1.** *A*, 293 cells were transfected with the indicated expression plasmids, and lysates were immunoprecipitated (*IP*) with anti-FLAG antibodies. After elution with a FLAG peptide, the material was immunoprecipitated with anti-TRAF3 antibodies. Anti-Myc Western analyses (*WB*) on final immunoprecipitates were carried out (*top*). *B*, LPS enhances association of TRAF3 with TBK1. RAW 264.7 cells were left untreated or stimulated with LPS for the indicated periods of time, and anti-TRAF3 immunoprecipitations followed by anti-TBK1 Western analyses were carried out (*top*). Anti-TBK1, -TRAF3, and IκBα Western analyses were performed on the cell extracts as well (*bottom*).

polyubiquitination occurred through a lysine-dependent mechanism, since no polyubiquitin-conjugated TANK adducts were detected using the HA-Ub (K0) mutant (Fig. 6*A*, *top*, *lane 4*). A similar experiment was conducted by overexpressing TBK1, the other LPS-inducible and TANK-phosphorylating kinase, and a TBK1-dependent TANK polyubiquitination was detected as well (Fig. 6*B*, *top*, *lane 3*). TANK was the targeted substrate for polyubiquitination as evidenced by the detection

FIGURE 3. **A TANK-containing immune complex phosphorylates IRF3/7 in LPS-stimulated macrophages.** *A*, endogenous TANK and IRF3 physically interact in LPS but not in TNFα-stimulated macrophages. RAW 264.7 cells were left untreated or stimulated with LPS (100 ng/ml) or TNFα (100 units/ml) for the indicated periods of time (*left* and *right*, respectively), and cell extracts were subjected to anti-TANK immunoprecipitations (*IP*) followed by anti-IRF3 Western analysis (*WB*) (*top*). Cell extracts were subjected to anti-TANK, -IRF3, and -Iκbα Western blots as well (*bottom*). *B* and *C*, IRF7 phosphorylation in LPS-stimulated (*B*) but not in TNFα-stimulated (*C*) RAW 264.7 macrophages by a TANK-containing complex. RAW 264.7 cells were left untreated or stimulated for the indicated periods of time with LPS or TNFα (*top*). Anti-TANK (*lanes 2–7*) or anti-HA (negative control; *lane 1*) immunoprecipitates were subjected to anti-IKKε or -TBK1 Western analysis (*second* and *third panels* from the *top*, respectively) or to *in vitro* kinase assays using the purified GST-IRF7 as substrate (*top*). Cell extracts were subjected to anti-TANK or IκBα Western analysis (*bottom*) as well. *D*, same experiment as in *A*, except that RAW 264.7 cells were replaced by peritoneal macrophages. An anti-TBK1 Western blot was used for normalization purposes (*middle*). *E*, TANK-depleted macrophages harbor impaired LPS-mediated IRF7 phosphorylation. CD14-stably expressing THP1 cells were infected with a lentiviral construct targeting either green fluorescent protein (negative control) or the TANK transcript, and the resulting cells were left unstimulated or treated with LPS for the indicated periods of time. Lysates were subjected to anti-TBK1 immunoprecipitations followed by *in vitro* kinase assays using a purified GST-IRF7 as substrate (*top*). *TBK1*^{P}, autophosphorylated TBK1. Cell extracts were subjected to anti-TANK, -TBK1, and -IκBα Western analyses as well. *F*, IRF7 is phosphorylated by a TANK-containing complex in CpG-stimulated macrophages. RAW 264.7 cells were left untreated or stimulated with CpG (1 μM) for the indicated periods of time, and anti-TANK immunoprecipitates were subjected to *in vitro* kinase assays using a purified GST-IRF7 as substrate (*top*). A positive control from LPS-stimulated cells is shown on *lane 1*. Anti-TANK and -IκBα Western analyses were carried out on the lysates (*bottom*). *G*, TANK is part of an IRF3-phosphorylating complex upon LPS or poly(I:C) stimulation in macrophages. RAW 264.7 cells were left unstimulated or treated with LPS (10 μg/ml) or poly(I:C) (100 μg/ml) (*left* or *right panel*, respectively) for the indicated periods of time, and anti-TANK or -TBK1 immunoprecipitates (*top panels* and *second panels* from the *top*, respectively) were subjected to *in vitro* kinase assays using the purified GST-IRF3 as substrate. Anti-TANK, -TBK1, or -IκBα Western analysis was carried out on the cell extracts as well (*bottom*).



of these polyubiquitin-conjugated adducts when the anti-FLAG immunoprecipitates were subjected to anti-TANK and -HA but not anti-Myc Western analyses (Fig. 6*C*, *lanes 3*, compare the *top* and *second panel versus* the *third panel* from the *top*). We previously reported that IKKβ is also a TANK kinase that targets the C-terminal part of this scaffold protein (37). We therefore tested whether IKKβ overexpression also triggers TANK polyubiquitination and concluded that, in contrast to ectopically expressed IKKε, this was not the case (Fig. 6*D*, *top*, *lanes 3* and *5*, respectively). IKKε-mediated TANK polyubiquitination required binding of the two proteins, since no polyubiquitin-conjugated TANK adducts were detected when IKKε ΔC30, which does not interact with TANK (Fig. 2*B*), was overexpressed (Fig. 6*E*, *top*, compare *lanes 3* and *4*). We next asked whether IKKε kinase activity was required in this process by testing the ability of an IKKε kinase-dead version ("IKKε KD") to trigger TANK polyubiquitination. Although overexpressed wild type IKKε phosphorylated and polyubiquitinated TANK (Fig. 6*F*, *lane 3*, *bottom* and *top panels*, respectively), IKKε KD, which indeed did not phosphorylate TANK, still triggered its polyubiquitination (Fig. 6*F*, *bottom* and *top panels*, respectively, *lane 4*). Thus, IKKε kinase activity is dispensable for TANK polyubiquitination. In conclusion, because TANK polyubiquitination is detected even in the absence of IKKε-mediated phosphorylation, our results suggest that the LPS-inducible TANK phosphorylation and the IKKε-mediated TANK polyubiquitination occur through independent mechanisms. We next stimulated macrophages with LPS, which triggers TBK1-IKKε activation and indeed detected endogenous polyubiquitinated adducts of TANK upon 15 min of stimulation (Fig. 6*G*, *top*, *lanes 2* and *3*).

TANK harbors a TRAF2/3 interaction domain located downstream from the TBK1-IKKε-interacting region (12, 18). To determine whether this domain is required for IKKε-mediated TANK polyubiquitination, we generated a mutant lacking this region ("TANKΔTRAF") and confirmed that this mutant indeed failed to interact with endogenous TRAF3, whereas wild type TANK did (Fig. 7*A*, *top*, compare *lanes 4* and *2*, respectively). Although a TANK mutant lacking the TBK1-IKKε-interacting site was not subject to IKKε-mediated polyubiquitination, the TANKΔTRAF mutant was still polyubiquitinated upon IKKε overexpression (Fig. 7*B*, *top*, *lanes 4* and *5*, respectively). This result demonstrates that TANK polyubiquitination requires binding to TBK1-IKKε but does not require the TRAF3-interacting site. Still, this result does not rule out the possibility that TRAF3 may be required for TANK polyubiquitination. To address this issue, we depleted TRAF3 in THP1/CD14 cells through RNA interference and assessed LPS-mediated TANK phosphorylation and polyubiquitination in these cells. As expected, LPS-mediated TANK phosphorylation was observed in THP1/CD14 cells infected with the shRNA control vector (Fig. 7*C*, *third panel* from the *top*, compare *lanes 6* and *7* with *lane 5*). Moreover, TANK polyubiquitination was also detectable after 15 min of stimulation (Fig. 7*C*, *top*, *lane 6*). Interestingly, TRAF3 depletion impaired both LPS-mediated TANK phosphorylation and polyubiquitination (Fig. 7*C*, *third panel* from the *top*, *lanes 2* and *3*, and *top panel*, *lane 2*, respectively). Thus, our results suggest that TRAF3 is essential for the LPS-mediated post-translational modifications of TANK, even if a direct interaction of TRAF3 with TANK is not required.

In summary, our observations suggest that the IRF3/7- and TANK-phosphorylating TBK1-IKKε kinases are necessary for the polyubiquitination of their scaffold protein TANK in the LPS-dependent pathway. Our data also highlight the critical role of TRAF3 in that pathway.

TBK1-IKKε Triggers TANK Lys^{63}-linked Polyubiquitination—By analogy with NEMO/IKKγ, which is subject to Lys^{63}-linked polyubiquitination, and because a prior phosphorylation does not appear to be required in that process, we hypothesized that TANK may be subjected to a similar post-translational modification. To test this possibility, we looked for polyubiquitin-conjugated TANK adducts in the presence of overexpressed HA-IKKε and Myc-tagged wild type or mutated (Lys^{48}, Lys^{63}, or both) Ub products (Fig. 8*A*). As expected, TANK was subjected to IKKε-mediated polyubiquitination in the presence of wild type Ub (Fig. 8*A*, *top*, *lane 3*). Polyubiquitinated TANK was also detected when the K48R but not the K63R or the K48R/K63R Ub mutant was co-transfected (Fig. 8*A*, *lanes 4–6*). Thus, IKKε triggers TANK polyubiquitination through a Lys^{63}-linked pathway. When these experiments were repeated using various TANK constructs lacking the N- or C-terminal domain, they revealed that the targeted residues on TANK are located between amino acids 71 and 110 (Fig. 8*B*, *top*, *lanes 4* and *5*). Moreover, a TANK mutant ("TANKΔC178") lacking the C-terminal inhibitory domain (12) was even more strongly polyubiquitinated (Fig. 8*B*, *top*, *lane 6*) through a Lys^{63} linkage (Fig. 8*C*, *top*, *lane 3*). This pathway occurred independently of the IKKε-mediated TANK phosphorylation process. Indeed, polyubiq-

FIGURE 5. **LPS triggers TANK phosphorylation in macrophages.** *A*, LPS-mediated TANK phosphorylation. RAW 264.7 cells were left unstimulated (*lane 1*) or treated with increasing concentration of LPS for the indicated periods of time, and cell extracts were subjected to anti-TANK and -IκBα Western analyses (*top* and *bottom*, respectively). *TANK^{P}*, phosphorylated TANK. *B*, LPS but not TNFα-mediated TANK phosphorylation in CD14-stably expressing THP1 cells. These cells were left untreated (*lanes 1*) or stimulated with LPS (100 ng/ml) or TNFα (100 units/ml) (*lanes 2–5*, *left* and *right*, respectively), and cell extracts were subjected to anti-TANK, -IκBα, and -TBK1 Western analyses. *C*, λ-phosphatase dephosphorylates TANK from LPS-stimulated macrophages. RAW 264.7 cells were left unstimulated (*lane 1*) or treated with LPS for 15 min (*lanes 2* and *3*), and the extracts were untreated (*lanes 1* and *2*) or incubated with 400 units of phosphatase (*lane 3*) at 37 °C for 30 min and subsequently subjected to anti-TANK Western analyses. *D*, staurosporin prevents LPS-mediated TANK phosphorylation. RAW 264.7 macrophages were preincubated with vehicle (Me_2SO (*DMSO*); *lanes 1–4*) or staurosporin (250 nM; *lanes 5–8*) for 20 min and subsequently unstimulated (*lanes 1* and *5*) or treated with LPS for the indicated periods of time (*lanes 2–4* and *6–8*). Cell lysates were subjected to anti-TANK, -IκBα, and -TBK1 Western analyses (*WB*). *E*, IKKε and TBK1 interact and phosphorylate TANK on identical domains. On the *left*, a schematic representation of the TANK constructs tested for interaction with TBK1-IKKε and phosphorylation by these kinases is shown. The mapping of the IKKε- and TBK1-interacting domains of TANK and the targeted domain of TANK for IKKε- and TBK1-mediated phosphorylation is illustrated. HeLa cells were transfected with the indicated expression plasmids, and anti-FLAG immunoprecipitates were subjected to *in vitro* kinase assays (*top panels*) or to anti-Myc western analyses (*second panel* from the *top*). Cell lysates were subjected to anti-FLAG or -Myc Western analyses as well (*bottom panels*). *F*, IRF3 and TANK are phosphorylated by TBK1 *in vitro*. On the *left*, a Coomassie Blue-stained gel showing the purified GST-IRF3 or -TANK fusion protein used as substrate in the *in vitro* kinase assay. On the *right*, the GST-IRF3 (*lanes 1* and *2*) and -TANK (*lanes 3* and *4*) were subjected to an *in vitro* kinase assay in presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of the recombinant TBK1 kinase. The figure shows the phosphorylated IRF3 and TANK proteins as well as the autophosphorylated TBK1 kinase.

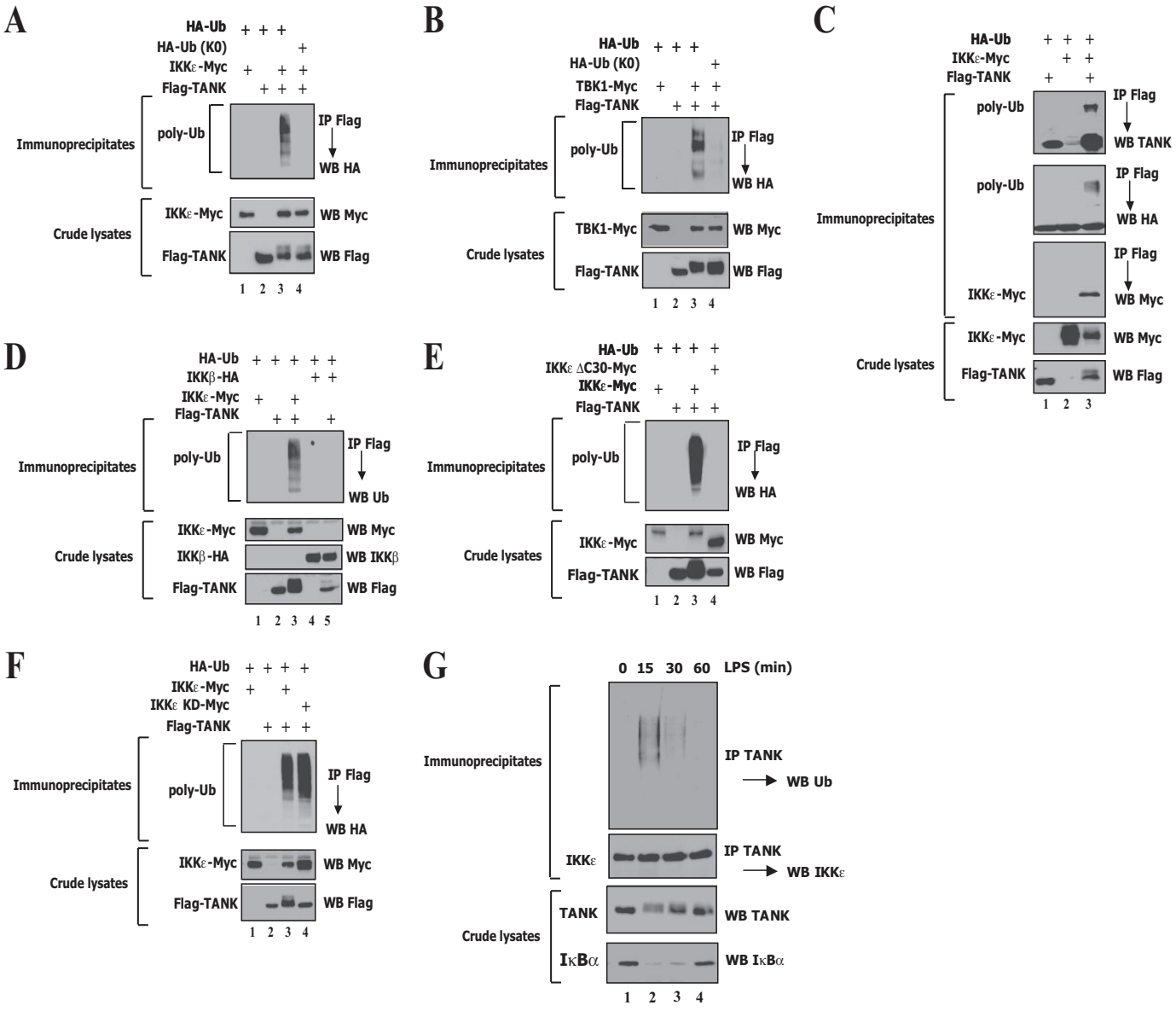


FIGURE 6. **TANK is subject to LPS and TBK1-IKKε-mediated polyubiquitination in macrophages.** *A–D*, overexpressed IKKε (*A* and *C*) or TBK1 (*B*) but not IKKβ (*D*) causes TANK polyubiquitination. 293 cells were transfected with the indicated expression plasmids, and cell extracts were subjected to anti-FLAG immunoprecipitations (*IP*) followed by an anti-HA (*A* and *B*) or -Ub (*D*) Western blot (*WB*) to detect the polyubiquitinated forms of TANK (*top*). Cell extracts were subjected to anti-Myc, -FLAG, and -HA (only in *D*) Western analysis as well (*middle* and *bottom*, respectively). *Poly-Ub*, polyubiquitination. *C*, anti-FLAG immunoprecipitates were also subjected to anti-TANK or -Myc Western analyses (*second* and *third panels* from the *top*, respectively). *E* and *F*, the C-terminal TANK-interacting region (*E*) but not the kinase domain of IKKε (*F*) is required for TANK polyubiquitination. 293 cells were transfected with the indicated expression plasmids, and the extracts were subjected to anti-FLAG immunoprecipitations followed by anti-HA Western analyses (*top*). Cell extracts were subjected to anti-Myc and -FLAG Western blots as well (*middle* and *bottom*, respectively). *G*, polyubiquitination of TANK upon LPS stimulation in macrophages. RAW 264.7 cells were untreated or stimulated with LPS (1 μg/ml) for the indicated periods of time, and cell extracts were subjected to anti-TANK immunoprecipitations followed by anti-ubiquitin Western analyses (*top*). As a loading control, the membrane was subsequently stripped and reprobed with the anti-IKKε antibody (*second panel* from the *top*). Cell extracts were subjected to anti-TANK and -IκBα Western analyses as well (*bottom*).

uitination was not detectable on the TANK ΔN110 mutant, which is still subjected to IKKε-mediated phosphorylation (see Fig. 5*E*, *top*, *lane 5*), whereas the −ΔC234 mutant, which is not subjected to IKKε-mediated phosphorylation (Fig. 5*E*, *top*, *lane 8*), was polyubiquitinated upon IKKε overexpression (Fig. 8, *B* and *D*).

Ubc13 is an E2 protein required for Lys^{63}-linked polyubiquitination (45). We tested IKKε-mediated TANK polyubiquitination in Ubc13-depleted cells and found that this process was severely impaired (Fig. 8*E*, *top*, *lanes 3* and *4*). Taken together, our results suggest that TANK is subject to Lys^{63}-linked polyubiquitination, which is dependent on TBK1-IKKε and Ubc13 and independent of TRAF and of IKKε-mediated phosphorylation.

DISCUSSION

We report here the identification of TANK/I-TRAF as the scaffold protein that assembles the TBK1-IKKε complex for subsequent IRF3/7 activations through some but not all TLR-dependent signaling pathways via binding to TRAF3. Impor-

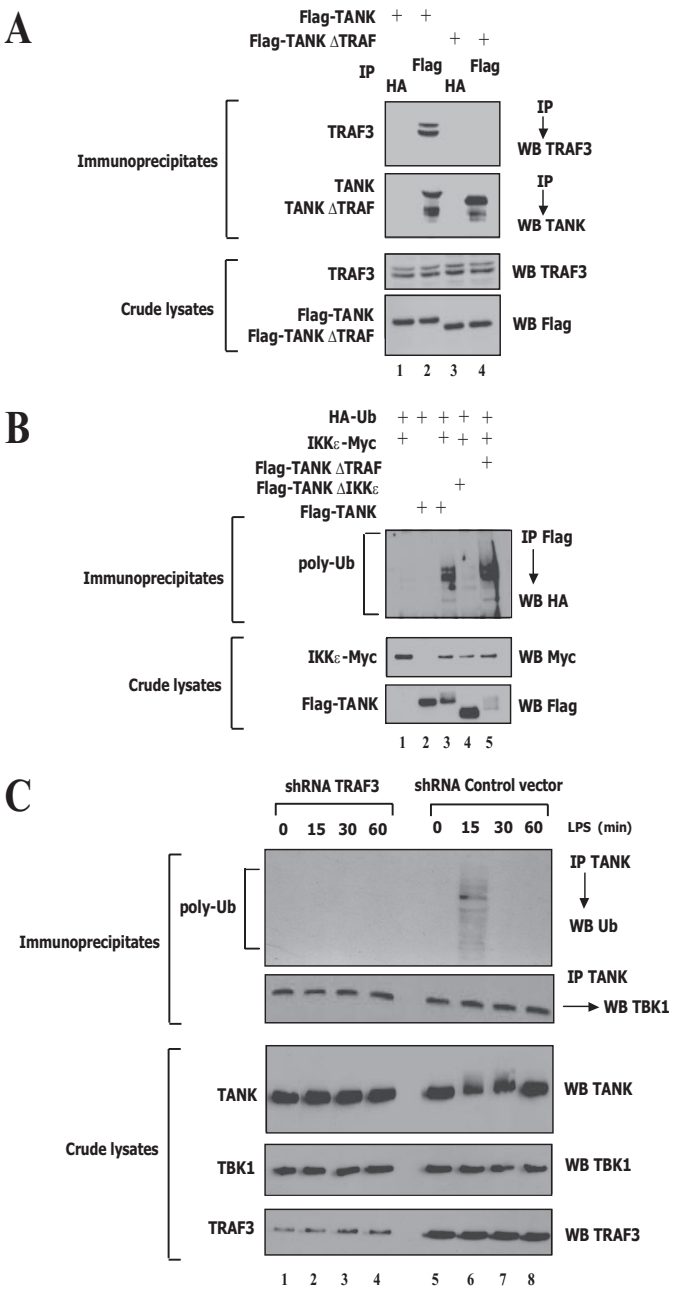
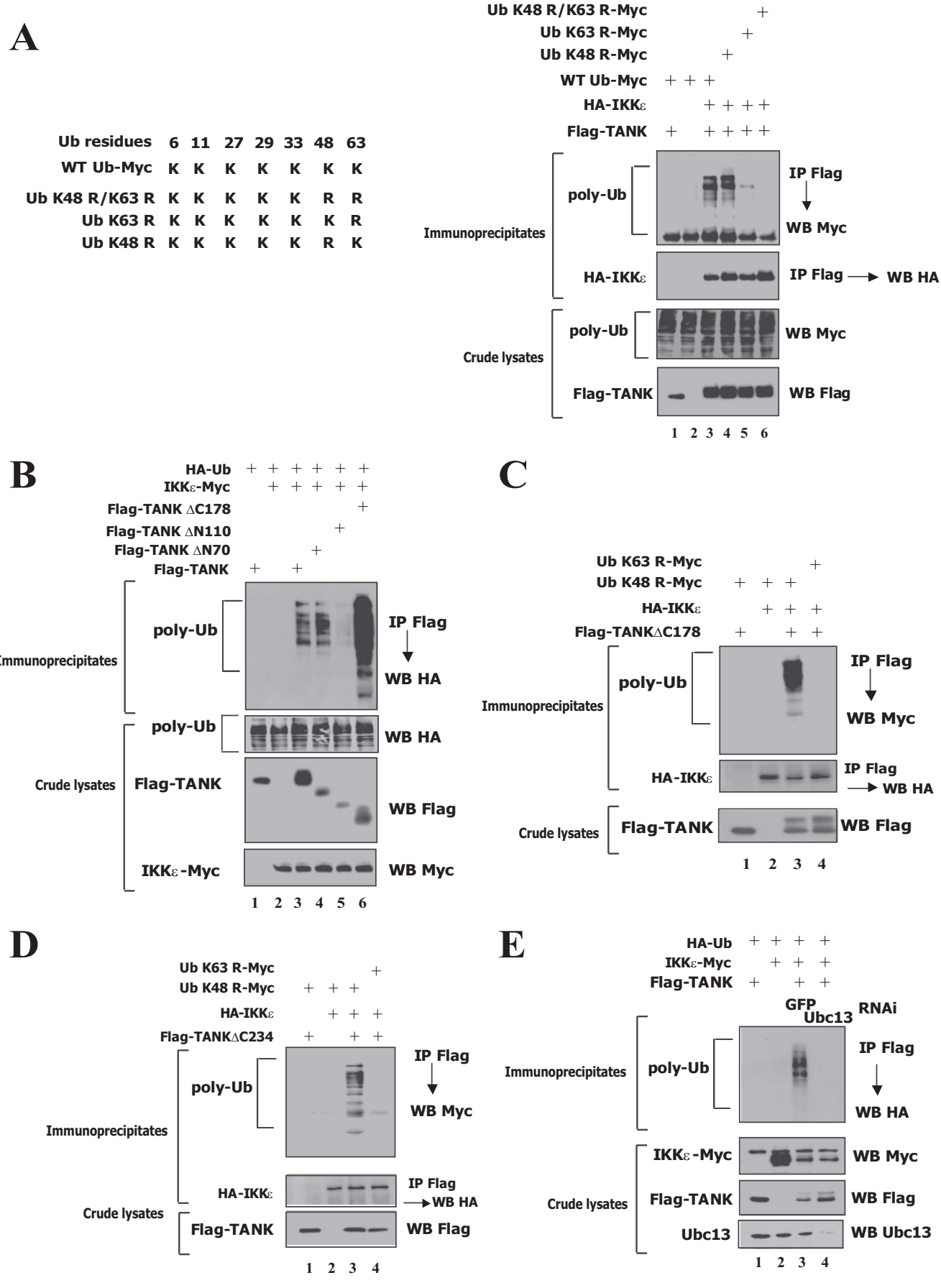


FIGURE 7. **IKKε-mediated TANK polyubiquitination occurs through a TRAF3-dependent pathway but does not require the TRAF3-interacting site on TANK.** *A*, TANKΔTRAF does not bind TRAF3. 293 cells were transfected with the indicated expression vectors, and cell extracts were subjected to anti-HA (negative control, *lanes 1* and *3*) or anti-FLAG immunoprecipitations (*IP*) (*lanes 2* and *4*) followed by anti-TRAF3 or -TANK analyses (*top* and *second panel* from the *top*, respectively). Cell extracts were subjected to anti-TRAF3 and -FLAG Western blots (*WB*) as well (*bottom*). *B*, both wild type TANK and TANKΔTRAF were subjected to IKKε-mediated polyubiquitination but not TANKΔIKKε. 293 cells were transfected with the indicated expression plasmids, and anti-FLAG immunoprecipitations followed by anti-HA Western analyses to detect TANK polyubiquitinated forms were carried out on the immunoprecipitates (*top*). Anti-Myc or -FLAG Western analyses were performed on the cell extracts as well (*bottom*). *C*, TRAF3 depletion impairs LPS-mediated TANK phosphorylation and polyubiquitination. THP1/CD14 infected with either the shRNA lentiviral construct targeting the TRAF3 transcript (*shRNA TRAF3*) (*lanes 1–4*) or the shRNA control vector (*lanes 5–8*) were left untreated (*lanes 1* and *5*) or stimulated with LPS (100 ng/ml) for the indicated periods of time. Anti-TANK immunoprecipitates were subjected to anti-ubiquitin Western analyses to detect TANK polyubiquitination (*top*), whereas cell extracts were subjected to anti-TANK, -TBK1, and -TRAF3 Western blots as well (*bottom*).

tantly, we also demonstrate that TANK undergoes two distinct post-translational modifications, namely phosphorylation and Lys^{63}-linked polyubiquitination, both of which require distinct functional domains of TBK1-IKKε.

TBK1-IKKε are IRF3/7 kinases, but how these phosphorylating complexes are assembled has hitherto been unknown. Here we show that TANK is required for both LPS and CpG-mediated IRF3/7 phosphorylations. The observation that TANK occurs in a triple complex with both TRAF3 and TBK1 combined with its inability to interact with TRAF6[9] or TAK1 (37) does not support a role for TANK in the Myd88- and TRAF6-dependent pathway of IKK activation. This conclusion is supported by the absence of an effect of TANK depletion on LPS-mediated IκBα degradation. Our results rather suggest that TANK assembles TBK1-IKKε downstream of TRAF3 for subsequent induction of IFN and potentially other target genes in LPS and also CpG-stimulated macrophages. The lack of association of TANK with TRIF, which is required for both the LPS- and the dsRNA-mediated IRF3 activations (34, 46), suggests that TANK may not act downstream of TRIF for IRF3 phosphorylation. However, such interactions were investigated in unstimulated cells and should thus be reevaluated in cells treated with the appropriate stimulus. Additional and still uncharacterized signaling molecules and/or post-translational modifications of TRAF3 or TANK may be required for proper assembling of these signaling complexes. Alternatively, a different scaffold protein may be required to assemble the IRF3 phosphorylating complex, and if so, NAP1 would be the most likely candidate. Indeed, this protein, initially identified as a TBK1/NAK-binding protein (47), was proposed to be the TBK1 scaffold protein acting in the TLR3-dependent pathway based on the defects seen in dsRNA-mediated IRF3 phosphorylation upon NAP1 depletion (11). Although we show here that a TANK-containing immune complex can phosphorylate IRF3 upon dsRNA stimulation in macrophages, such phosphorylation only occurs with delayed kinetics (60 min), which may reflect indirect mechanisms. Such an observation strongly suggests that another scaffold protein, most likely NAP1, is required for early IRF3 phosphorylation through the TLR3-dependent pathway. Thus, our present report suggests that TBK1-IKKε may be assembled by distinct but structurally similar scaffold proteins in a pathway-specific manner. In other words, TANK/I-TRAF would be the candidate that assembles the TBK1-IKKε complex upon LPS stimulation, whereas NAP1 would be the one in dsRNA-treated cells. This phenomenon has already been demonstrated for the signaling cascades that make use of distinct IKK complexes for NF-κB activation. Indeed, whereas NEMO/IKKγ is essential for the IKKα/β-mediated NF-κB activation through the so-called classical pathway, this scaffold protein is dispensable for the alternative pathway of NF-κB activation, which relies on an unidentified IKKα adapter (48). We therefore propose the existence of multiple pools of TBK1-IKKε assembled by distinct scaffold proteins, which endow specificity in the TLR-dependent signaling pathways.

[9] T.-L. Chau and A. Chariot, unpublished results.



A role for TANK/I-TRAF in the TLR-dependent signaling pathways does not rule out the possibility that the TLR-independent pathways triggered upon viral infection, such as the one that relies on retinoic acid-inducible gene I, for example, also involve this scaffold protein, and a couple of recently published studies actually provided experimental evidence for this (49, 50).

We demonstrate here that TANK/I-TRAF is subject to post-translational modifications, namely phosphorylation and Lys^{63}-linked polyubiquitination, both of which require TBK1-IKKε. TANK phosphorylation is intimately linked to IRF3/7 phosphorylations through both the TLR4 and the TLR9 pathways. Interestingly, the kinetics of TANK phosphorylation is stimulus-dependent, since it occurs after 15 or 60 min of LPS or poly(I:C) stimulations, respectively. Several hypotheses, which all require experimental validations, may explain this shifted kinetics. Besides the possibility that distinct scaffold proteins may assemble the TBK1-IKKε complexes, distinct and yet to be characterized upstream signaling molecules may be involved in this TANK post-translational modification. In this context, whether Myd88 is involved for TANK phosphorylation may be signal-specific. Indeed, whereas the LPS-mediated IRF3 activation relies on the TIR domain-containing adaptors TRIF-related adaptor molecule and TRIF but not on Myd88, the CpG-mediated IRF7 activation requires Myd88 (51). Therefore, and because both pathways ultimately trigger TBK1-IKKε activations and TANK phosphorylation, as shown here, this opens the possibility that TBK1-IKKε kinase activity may be induced through recruitment of distinct upstream signaling complexes in a signal-specific manner. The delayed kinetics seen for TANK phosphorylation upon dsRNA stimulation may also be due to a transcriptionally inducible expression of IKKε but not of TBK1, which implies that TANK would be preferentially targeted for phosphorylation by TBK1 in early phases and subsequently by IKKε in latter times. In any case, whether such post-translational modification of TANK is required for TBK1-IKKε activation is currently unclear. Remarkably, although LPS and CpG-mediated TANK phosphorylations require TBK1-IKKε kinase function, LPS-mediated Lys^{63}-linked TANK polyubiquitination does not. TANK mutants lacking the residues phosphorylated by TBK1-IKKε are still able to undergo Lys^{63}-linked polyubiquitination, suggesting that the two modifications occur independently. Therefore, TBK1-IKKε harbor a previously undescribed function, namely the ability to trigger non-degradative polyubiquitination of their scaffold protein TANK. Our results also suggest that nondegradative polyubiquitination may critically regulate the TLR-dependent signaling cascades, leading to IFN type I gene induction.

TRAF6 harbors a so-called RING domain and possesses intrinsic ubiquitin ligase activity, which ultimately targets NEMO/IKKγ for polyubiquitination upon IL-1β stimulation (52). Although TRAF3 harbors a RING domain, which is essential for suppression of the noncanonical NF-κB pathway (53), it is currently unclear whether TRAF3 also acts as an E3 ligase similarly to TRAF6. We demonstrate here that TRAF3 is required for LPS-mediated TANK phosphorylation but also for TANK polyubiquitination. Interestingly, TRAF3 does not have to bind TANK for this polyubiquitination to occur. Therefore, TRAF3 may recruit a yet to be identified LPS-inducible E3 ligase to TANK for subsequent polyubiquitination of this scaffold protein. Ongoing experiments are dedicated to the identification of this candidate.

NEMO/IKKγ is the best known example of a scaffold protein subject to Lys^{63}-linked polyubiquitination in response to several stimuli, and this post-translational modification appears to be essential for IKK and subsequent classical NF-κB activation. The Lys^{63}-linked polyubiquitination of TANK requires TBK1-IKKε and the E2 protein Ubc13 but is TRAF-independent. Even if some analogy with NEMO/IKKγ can therefore be established, some differences persist. Unlike TANK, which requires binding to TBK1-IKKε, NEMO/IKKγ Lys^{63}-linked polyubiquitination does not appear to require IKKα/β, the kinase subunits that NEMO/IKKγ assembles. Although it is unclear how Lys^{63}-linked TANK polyubiquitination regulates IRF3/7 activations, it is noteworthy that the N-terminal part of TANK, which has been described as positively regulating signal transduction (12), harbors the TBK1-IKKε-interacting site and is also very strongly Lys^{63}-linked polyubiquitinated. It may be speculated that this polyubiquitin linkage of TANK, which does not require prior phosphorylation, positively regulates IRF3/7 activation. In this context, a recent report demonstrated that Lys^{63}-linked polyubiquitination of retinoic acid-inducible gene I by the E3 ligase TRIM25 is critical for the retinoic acid-inducible gene I-dependent antiviral signal transduction (54). Because this latter pathway also involves IRF activation, it is tempting to speculate that optimal activation of these pathways relies on signal-specific E3 ligases whose identities remain unknown in most cases so far.

Recent reports revealed that TBK1 and IKKε have unexpected physiological functions, namely a role in angiogenesis (55) and in linking innate immune signaling and tumor cell survival through a RalB GTPase pathway (for TBK1) (56) as well as a role in actin cytoskeleton organization in *Drosophila* (for IKKε) (57). The upstream signaling molecules such as TRAF3 and TRIF and the scaffold proteins TANK and NAP1 and their regulatory involvement in these new TBK1-IKKε-dependent pathways is, as yet, unknown. These reports combined with our study show that a more complete understanding of the varied roles of TBK1-IKKε requires a thorough identification of their substrates and the characterization of their kinase-independent functions.

FIGURE 8. **IKKε-mediated TANK polyubiquitination occurs through an Ubc13-dependent Lys^{63} linkage and does not require prior phosphorylation by that kinase.** *A–D*, overexpressed IKKε causes a Lys^{63}-linked TANK polyubiquitination through a phospho-independent mechanism. 293 cells were transfected with the indicated expression plasmids, including wild type or Myc-tagged ubiquitin mutants (*A*, *C*, and *D*), whose sequences are illustrated on the *left* (*A*). Anti-FLAG immunoprecipitates (*IP*) were subjected to anti-Myc Western blots (*WB*) (*A*, *C*, and *D*) and also to anti-HA Western analyses to detect either polyubiquitinated adducts of TANK (*B*) (*top*) or HA-tagged IKKε (*middle panels* in *A*, *C*, and *D*). Anti-FLAG (*A–D*) or -anti-Myc (*A* and *B*) Western blots were also performed with the extracts (*bottom*). *E*, Ubc13 is required for IKKε-mediated TANK polyubiquitination. 293 cells were transfected with RNA interference targeting either green fluorescent protein (negative control) or the Ubc13 transcript, and the resulting cells were transfected with the indicated expression plasmids. Detection of polyubiquitinated forms was carried out after immunoprecipitation as described here before (*top*), and extracts were subjected to anti-Myc, FLAG, or Ubc13 Western analyses as well (*bottom*).

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