The Journal of Immunology

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J Immunol 2011; 186:499-507; Prepublished online 24 November 2010;
doi: 10.4049/jimmunol.0903534
http://www.jimmunol.org/content/186/1/499

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Akt Contributes to Activation of the TRIF-Dependent Signaling Pathways of TLRs by Interacting with TANK-Binding Kinase 1

Sun Myung Joung,*,† Zee-Yong Park,* Shilpa Rani,* Osamu Takeuchi,‡ Shizuo Akira,‡ and Joo Young Lee*,†

Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) is an adaptor molecule that is recruited to TLR3 and -4 upon agonist stimulation and triggers activation of IFN regulatory factor 3 (IRF3) and expression of type 1 IFNs, which are critical for cellular antiviral responses. We show that Akt is a downstream molecule of TRIF/TANK-binding kinase 1 (TBK1) and plays an important role in the activation of IRF3 by TLR3 and -4 agonists. Blockade of Akt by a dominant-negative mutant or by short interfering RNA decreased IRF3 activation and IFN-β expression induced by polyinosinic:polycytidylic acid [poly(I:C)], LPS, TRIF, and TBK1. Association of endogenous TBK1 and Akt was observed in macrophages when stimulated with poly(I:C) and LPS. In vitro kinase assays combined with reversed-phase liquid chromatography mass spectrometry analysis showed that TBK1 enhanced phosphorylation of Akt on Ser^{473}, whereas knockdown of TBK1 expression by short interfering RNA in macrophages decreased poly(I:C)- and LPS-induced Akt phosphorylation. Embryonic fibroblasts derived from TBK1 knockout mice also showed impaired Akt phosphorylation in response to poly(I:C) and LPS. To our knowledge, our results demonstrate a new regulatory mechanism for Akt activation mediated by TBK1 and a novel role of Akt in TLR-mediated immune responses. ***The Journal of Immunology*****, 2011, 186: 499–507.**

Toll-like receptors play an essential role in host defense against invading microbial pathogens by regulating innate and adaptive immune responses (1, 2). Activation of TLRs by agonists triggers the recruitment of adaptors leading to activation of two major downstream signaling pathways: the MyD88-dependent and -independent (Toll/IL-1R domain-containing adaptor inducing IFN-β [TRIF]-dependent) pathways (3). Although both pathways induce the activation of NF-κB though with different kinetics, the TRIF pathway is mainly responsible for TLR3 and -4–dependent IFN regulatory factor 3 (IRF3) activation. TANK-binding kinase 1 (TBK1) and inhibitor of κB kinase ε, which in turn phosphorylate and activate IRF3, an important transcription factor for antiviral cellular responses involving the production of type I IFNs (4–6). The expression of >70% of LPS (a TLR4 agonist)-induced genes is known to be regulated via the TRIF pathway (7), and TLR3 activation is primarily dependent on activation of this pathway. These facts underline the critical role of TRIF-dependent signaling in TLR3 and -4–mediated immune regulation.

Akt, also called protein kinase B, is a serine/threonine kinase that is implicated in a variety of cellular responses and in the pathogenesis of many metabolic diseases and cancers. Activation of Akt is initiated by binding of its N-terminal pleckstrin homology (PH) domain to phosphatidylinositol-3,4,5-triphosphates produced by phosphoinositide 3-kinase (PI3K) and translocation of Akt to the plasma membrane (8). Akt is then, phosphorylated at Thr^{308} in the activation loop by phosphatidyl inositol-dependent kinase-1 and Ser^{473} in the hydrophobic motif of the C terminus by various kinases including the rictor–mammalian target of rapamycin complex (9). Akt is implicated as one of the downstream signaling components of TLRs; stimulation of cells with various TLR agonists induces the phosphorylation and activation of Akt, and the survival and maturation of bone marrow-derived dendritic cells stimulated with LPS is greatly impaired in Akt1-deficient cells (10). Downregulation of Akt activity using a dominant-negative mutant (DN) resulted in blockade of NF-κB activation induced by MyD88 suggesting a positive role of Akt in the MyD88-dependent pathway of TLRs (11). However, the role of Akt in the TRIF-mediated signaling pathway of TLRs is not entirely understood.

The aim of this study was to clarify the role of Akt in TRIF-mediated signaling of TLR3 and -4. The results of experiments described below suggest that TBK1 and Akt cooperate to induce maximal activation of IRF3 and expression of IFN-β.

Materials and Methods

Cell culture

RAW264.7 cells (the murine monocytic cell line, ATCC TIB-71, American Type Culture Collection, Manassas, VA), 293T (human embryonic kidney cells), and embryonic fibroblast cells from wild-type (WT; *Tbk1*$^{+/+}$ and

*School of Life Sciences and †Immune Synapse Research Center, Gwangju Institute of Science and Technology, Gwangju, Korea; and ‡Laboratory of Host Defense, Immunology Frontier Research Center, Osaka University, Osaka, Japan

Received for publication October 30, 2009. Accepted for publication October 24, 2010.

This work was supported by a grant from Cell Dynamics Research Center, the National Research Foundation of Korea funded by the Korean Government (MEST) (2010-0001617), and a grant from the National Research Foundation of Korea, MEST (20100000420).

Address correspondence and reprint requests to Dr. Joo Young Lee, School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju, Korea, 500-712. E-mail address: joolee@gist.ac.kr

The online version of this article contains supplemental material.

Abbreviations used in this paper: CA, constitutively active mutant; Cont, control; DN, dominant-negative mutant; IB, immunoblotted; IP, immunoprecipitated; IRF3, IFN regulatory factor 3; MEF, mouse embryonic fibroblast; MS/MS, tandem mass spectra; ND, phospho-IRF3 or IRF3 dimer was not detected; PH, pleckstrin homology; poly(I:C), polyinosinic:polycytidylic acid; RPLC-MS/MS, reversed-phase liquid chromatography mass spectrometry; siRNA, short interfering RNA; TBK1, TANK-binding kinase 1; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; WCL, whole cell lysates; WT, wild-type.

Tbk1$^{+/-}$) and TBK1 knockout (*Tbk1*$^{-/-}$) mice were cultured in DMEM containing 10% (v/v) heat-inactivated FBS (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) (12, 13). Bone marrow-derived macrophages isolated from WT (C57BL/6) mice (Japan SLC, Shizuoka, Japan) were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 20% L929 cell-conditioned medium for 6 d, and adherent cells were used as macrophages (12).

Reagents

Purified LPS and polyinosinic:polycytidylic acid [poly(I:C)] were obtained from List Biological Laboratory (Campbell, CA) and Amersham Biosciences (Pittsburgh, PA), respectively. Anti-Akt Ab and anti-phospho (Ser^{473})-Akt Ab were purchased from Cell Signaling Technology (Beverly, MA). Anti-GFP Ab was from Invitrogen. Anti-TBK1 Ab and anti-flag Ab were from Imgenex (San Diego, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Anti-myc Ab and anti-actin Ab were purchased from Cell Signaling Technology and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Native alkaline phosphatase and staurosporine were from Calbiochem (San Diego, CA).

Plasmids

The IFN-β PRDIII-I-luciferase reporter plasmid, the expression plasmid for flag-tagged WT TBK1, TBK1(K38A), and the Gal4-IRF3 plasmid were kind gifts from Dr. Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA). The myc-tagged WT TBK1 plasmid was provided by Fumiyo Ikeda (Goethe University Medical School, Frankfurt, Germany). The heat shock protein 70-galactosidase reporter plasmid was from Dr. Robert Modlin (University of California, Los Angeles, Los Angeles, CA), and the TLR3 expression plasmid was obtained from Dr. Ruslan Medzhitov (Yale University, New Haven, CT). The DNs of p85 (p85ΔiSH2) and Akt (Akt T308A/S473A) were obtained from Dr. Bing-Hua Jiang (West Virginia University, Morgantown, VA), whereas WT Akt were provided by Dr. Michael Weber (University of Virginia Health Sciences Center, Charlottesville, VA). The expression plasmids of GFP-tagged Akt(WT), Akt(PH), and Akt(ΔPH) were provided by Dr. Scott A. Summers (Colorado State University, Fort Collins, CO) (14). The expression plasmids of GFP-IRF3(WT), GFP-IRF3(5D), and GFP-IRF3(5A) were provided by Dr. John Hiscott (McGill University, Montreal, Quebec, Canada). All DNA constructs were prepared for transfection using an EndoFree plasmid maxi-kit (Qiagen, Chatsworth, CA).

Transfection and luciferase assay

RAW264.7 or 293T cells were transfected with a luciferase plasmid and various expression plasmids of signaling components using SuperFect transfection reagent (Qiagen). Heat shock protein 70–β-galactosidase plasmid was cotransfected as an internal control. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer's instructions.

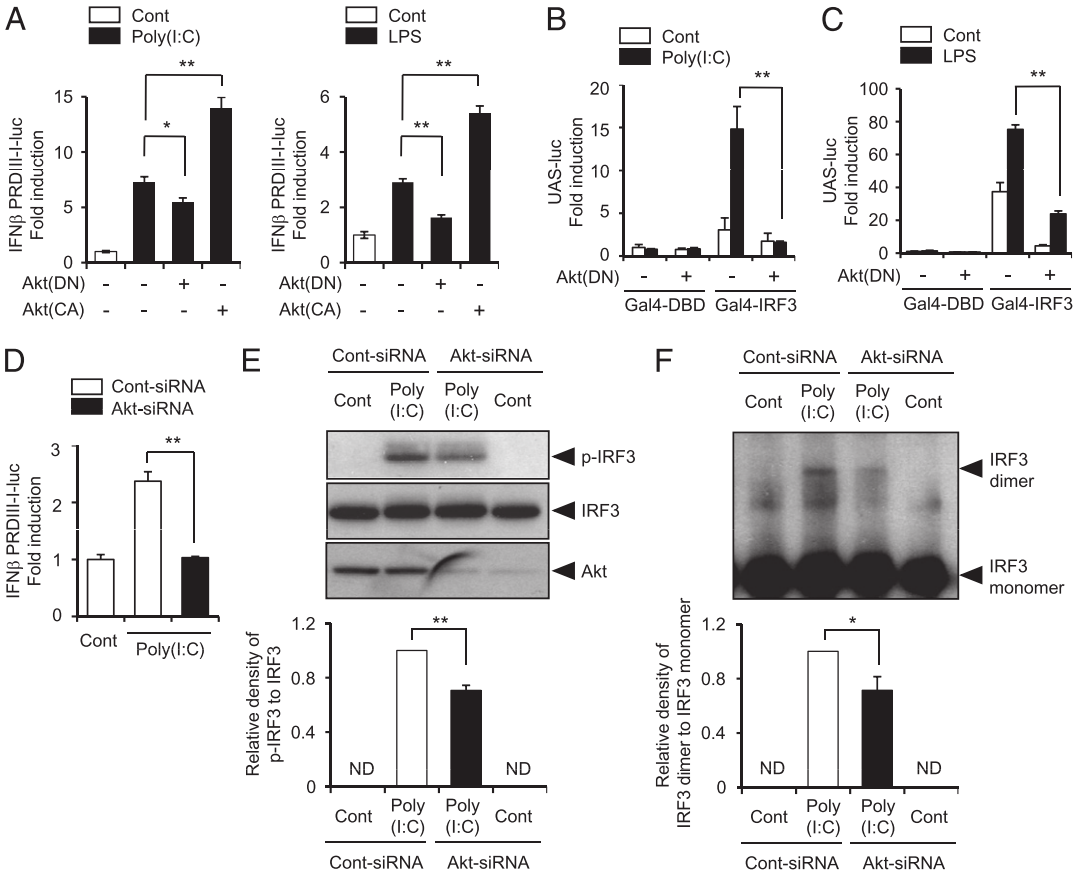

FIGURE 1. Akt is involved in IRF3 activation induced by TLR3 and -4 agonists. *A*, RAW264.7 cells were transfected with a luciferase reporter plasmid containing an IRF3 binding site (IFN-β PRDIII-I-luc) and an expression plasmid for Akt(DN) or Akt(CA) and incubated with poly(I:C) (30 μg/ml) or LPS (10 ng/ml) for 18 h. 293T cells expressing TLR3 (*B*) and RAW264.7 cells (*C*) were transfected with a luciferase reporter plasmid containing the Gal4 upstream activation sequence (UAS-luc) and an expression plasmid for Gal4-DBD (the yeast Gal4 DNA-binding domain), Gal4-IRF3 (Gal4-DBD fused to IRF3 lacking its own DNA-binding domain), or Akt(DN). They were then incubated with poly(I:C) (30 μg/ml) or LPS (10 ng/ml) for 18 h. *D*, 293T cells were transfected with IFN-β PRDIII-I-luc and an expression plasmid for TLR3 together with siRNA for Akt and further incubated with poly(I:C) (30 μg/ml) for 18 h. Data are presented as fold inductions (mean ± SEM, $n = 3$). *E* and *F*, Bone marrow-derived macrophages were transfected with siRNA for Akt and stimulated with poly(I:C) (30 μg/ml) for 1 h. Cell lysates were analyzed by immunoblotting for phospho(Ser^{396})-IRF3, IRF3, and Akt. The representative figures are presented out of three separate experiments. The relative densities of phospho-IRF3 and IRF3 dimer to IRF3 and IRF3 monomer, respectively, were quantified using densitometry and analyzed for statistical differences (mean ± SEM, $n = 3$). *$p < 0.05$; **$p < 0.01$. Cont, control; CA, constitutively active mutant; ND, phospho-IRF3 or IRF3 dimer was not detected.

Immunoprecipitation and immunoblotting

Supernatants from cell lysates were incubated with the primary Ab indicated in the figures and further incubated with protein A-agarose. After immune complexes were solubilized with Laemmli sample buffer, these were resolved on SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membranes were blotted with corresponding primary Ab and secondary Ab conjugated to HRP. The reactive bands were visualized with the ECL system.

Quantitative real-time PCR analysis

Total RNAs extracted using TRIzol reagent were reverse-transcribed with the UmProm-II Reverse Transcriptase (Promega, Madison, WI) and amplified with a Chromo4 (MJ Research, Waltham, MA) using SYBR Premix Ex Taq for quantitative real-time PCR analysis. The primers were: *Ifn-β*, 5′-TCCAAGAAAGGACGAACATTCG-3′ and 5′-TGCGGACATCTCCCACGTCAA-3′; *Rantes*, 5′-GCCCACGTCAAGGAGTATTTCTAC-3′ and 5′-AGGACTAGAGCAAGCGATGACAG-3′; and *β-actin*, 5′-TCATGAAGTGTGTTGACATCCGT-3′ and 5′-TTGCGGGCACGATGGAGGGGCCGGA-3′. The specificity of the amplified PCR products was assessed by a melting curve analysis. Fold inductions of gene expression were calculated as previously described (12).

RNA interference

Human Akt-specific short interfering RNA (siRNA) was purchased from Cell Signaling Technology. Mouse Akt1- and human p110α (PI3K catalytic subunit)-specific siRNAs were purchased from Dharmacon (Lafayette, CO) and TBK1-specific siRNAs from Invitrogen. Nontargeting control siRNA that does not match any human or mouse transcript was synthesized with the following sequence: 5′-GACUACUGGUCGUUGAACU-3′. Cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The results of target gene knockdown were shown in Supplemental Fig. 1.

ELISAs

Levels of IFN-β in culture media were determined using the ELISA kit (R&D Systems, Minneapolis, MN). The concentration ranges for the standard curves were from 15.6–1000 pg/ml. The samples were properly diluted to be measured within the standard curve range.

In vitro TBK1 kinase assay

TBK1 kinase assay was conducted with recombinant active human TBK1 (Millipore, Billerica, MA) according to the manufacturer's instruction with modification (15). Briefly, active TBK1 was incubated with recombinant inactive human Akt1 (20 ng; Millipore) in kinase buffer containing 50 μM ATP for 40 min at 30°C. Phosphorylation of Akt was determined by immunoblotting using anti-phospho(Ser^{473})-Akt Ab.

In-gel digestion, reversed-phase liquid chromatography mass spectrometry analysis, and relative quantification of phosphorylated peptides

The protein samples used in the in vitro TBK1 kinase assay were subjected to electrophoresis on 10% SDS polyacrylamide gels. These were stained with Coomassie Brilliant blue, and protein bands matching the m.w. of Akt were excised from the stained gel. The details of in-gel digestion procedure are described elsewhere (16). Briefly, proteins contained in the gel pieces were reduced with 2 mM tris(2-carboxyethyl)phosphine at room temperature for 45 min and then alkylated with 25 mM iodoacetamide at room temperature for 30 min. The gel pieces were soaked and incubated in sequencing-grade trypsin solution (500 ng) for 14–18 h at 37°C, and the tryptic peptides were extracted from the gel pieces and evaporated under vacuum. In-gel digested protein samples were analyzed by reversed-phase liquid chromatography mass spectrometry (RPLC-MS/MS) analysis using an LTQ linear ion trap mass spectrometer (Thermo Finnigan, Palo Alto, CA). Tandem mass spectra (MS/MS) were searched against an in-house protein database containing the Akt sequence using TurboSequest, and static modifications of cysteines (+57 on Cys) and differential modifications of serines, threonines, and tyrosines (+80 on Ser, Thr, Tyr) were considered in the search. The maximum number of modifications allowed per peptide was 7. Bioworks Ver 3.2 (Thermo Finnigan) was used to filter the search results, and the following Xcorr values were applied to the different charge states of peptides, and a δ Cn score of 0 was applied to all charge states: 1.8 for singly charged peptides, 2.2 for doubly charged peptides, and 3.2 for triply charged peptides. Manual validations were carried out on the filtered MS/MS spectra to confirm the database search results. The following criteria were used in the manual validation: 1) presence of major fragment ion series (b- and y-); 2) presence of strong neutral loss of phosphorylated group (−98 Da) in the case of Ser- and Thr-phosphorylated peptides; 3) presence of preferential cleavage products such as the N-terminal fragment ions of Pro and C-terminal fragment ions of D; and 4) presence of specific cleavage sites of trypsin. To monitor the level of Akt phosphorylation under various conditions, we constructed selected ion chromatograms of the Akt tryptic digest peptides (phosphorylated and unmodified forms). Each peak in the selected ion chromatograms was manually confirmed by the presence of the corresponding MS/MS spectra and phosphorylation sites. The peak area of each peptide was calculated with the built-in integration function of XCalibur 4.1 (Thermo Finnigan). The percentage of phosphorylation in Table I was defined as the percentage ratio between the integrated areas of the phosphorylated peptide and the integrated areas of the corresponding unmodified peptide.

Statistical analysis

Immunoblot data were presented with representative figures from more than three independent experiments. Other data were expressed as mean ± SEM. Difference between groups was examined by Student *t* test (significant when $p < 0.05$).

Results

Akt is required for IRF3 activation induced by TLR3 and -4 agonists

To investigate the role of Akt in TRIF-dependent signaling, first we determined if TLR3 and -4 agonists induce Akt activation. We found that incubation of RAW264.7 cells with poly(I:C) or LPS, as well as addition of poly(I:C) to 293T cells expressing TLR3, induced phosphorylation of Akt, thus demonstrating activation of Akt by TLR3 and -4 agonists (Supplemental Fig. 2). Next,

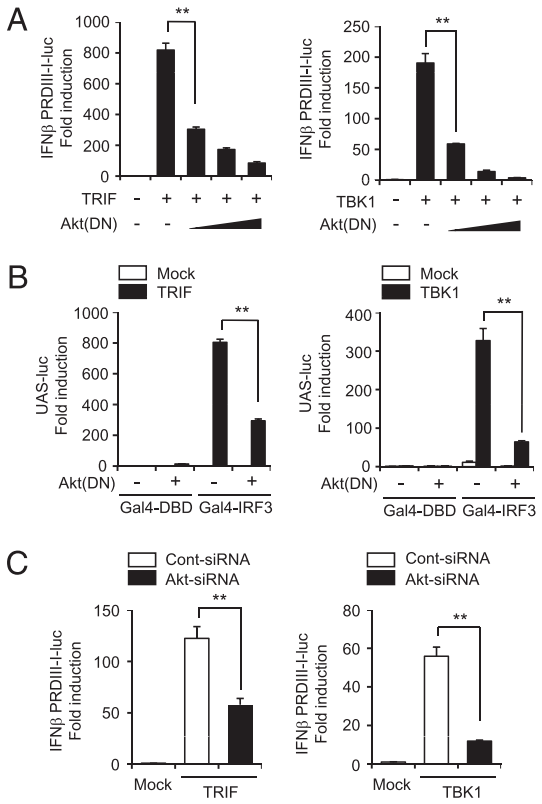

FIGURE 2. Akt is a downstream component of the TRIF/TBK1 pathway contributing to IRF3 activation. *A*, 293T cells were transfected with IFNβ PRDIII-I-luc and an expression plasmid for TRIF, TBK1, or Akt(DN). The expression of Akt(DN) was confirmed by immunoblotting (Supplemental Fig. 3). *B*, 293T cells were transfected with UAS-luc and an expression plasmid for Gal4-DBD, Gal4-IRF3, TRIF, TBK1, or Akt(DN). *C*, 293T cells were transfected with siRNA for Akt, IFN-β PRDIII-I-luc, and an expression plasmid for TRIF and TBK1. Luciferase activities were determined and are presented as fold inductions (mean ± SEM, $n = 3$). $^{**}p < 0.01$.

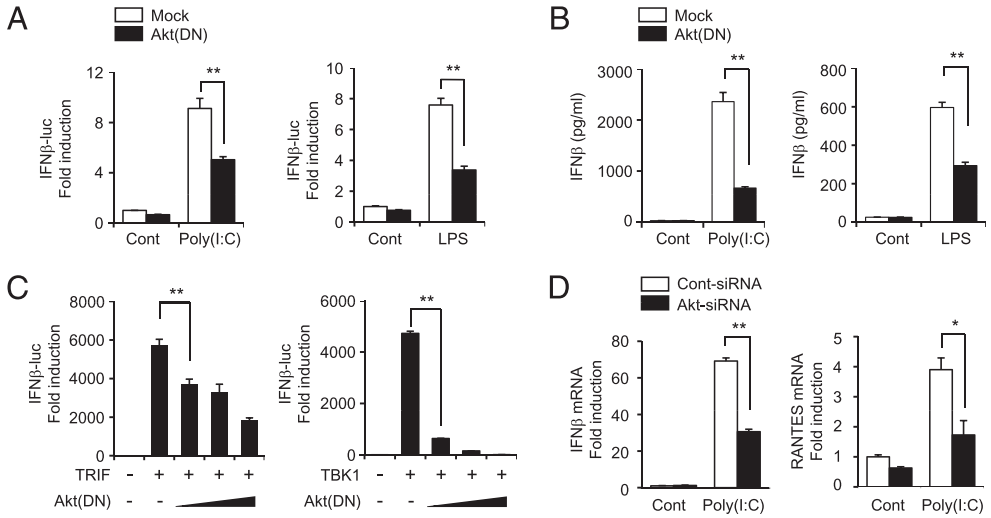

FIGURE 3. Akt is involved in IFN-β expression induced by activation of TLR3 and -4. *A*, RAW264.7 cells were transfected with a luciferase reporter plasmid containing the IFN-β promoter (IFNβ-luc) and an expression plasmid for Akt(DN) and incubated with poly(I:C) (30 μg/ml) or LPS (10 ng/ml) for 18 h. *B*, RAW264.7 cells were transfected with an expression plasmid of Akt(DN) and incubated with poly(I:C) (30 μg/ml) or LPS (10 ng/ml) for 18 h. The protein levels of IFN-β were determined by ELISA from cell supernatants. *C*, 293T cells were transfected with IFNβ-luc and an expression plasmid for TRIF, TBK1, or Akt(DN). Luciferase activities were determined and are presented as fold inductions. *D*, RAW264.7 cells were transfected with siRNA for Akt and further treated with poly(I:C) (30 μg/ml) for 3 h. Total RNAs were extracted, and levels of IFN-β and RANTES expression were determined by quantitative real-time PCR analysis; they are presented as fold inductions postnormalization with β-actin expression (mean ± SEM, $n = 3$). $*p < 0.05$; $**p < 0.01$.

we determined if IRF3 activation in response to these agonists is influenced by the Akt pathway. A DN of Akt suppressed poly(I:C)- or LPS-induced expression of an IRF3-dependent reporter, which consists of PRDIII and PRDI sites of IFN-β promoter region, whereas constitutively active Akt potentiated the IRF3-reporter expression (Fig. 1*A*). The transcriptional activity of IRF3 increased by poly(I:C) and LPS was also significantly decreased by Akt(DN) as determined by Gal4-IRF3 (Gal4 DNA-binding domain fused to IRF3 lacking its own DNA-binding domain) and Gal4 upstream activation sequence-luciferase system (Fig. 1*B*, 1*C*). Furthermore, knockdown of Akt expression using Akt siRNA blocked poly(I:C)-induced IRF3-reporter expression in 293T cells expressing TLR3 (Fig. 1*D*). IRF3 phosphorylation and dimerization induced by poly(I:C) were also attenuated by Akt siRNA in bone marrow-derived macrophages (Fig. 1*E*, 1*F*). These results show that Akt is involved in IRF3 activation induced by TLR3 and -4 agonists.

Akt is a downstream component of TRIF and TBK1

To identify the signaling step in which Akt is involved to contribute to IRF3 activation, we investigated if Akt plays a role as a downstream component of TRIF. Akt(DN) suppressed IRF3-reporter expression induced by TRIF (Fig. 2*A*, *left panel*), showing that Akt (DN) inhibits ligand-independent IRF3 activation. Akt(DN) also suppressed IRF3-reporter expression induced by TBK1, a downstream kinase of TRIF (Fig. 2*A*, *right panel*), suggesting that Akt works downstream of TBK1. These were consistent with the results that Akt(DN) suppressed IRF3 transcriptional activity increased by TRIF and TBK1 (Fig. 2*B*). In addition, Akt siRNA blocked IRF3-reporter expression induced by TRIF and TBK1 (Fig. 2*C*). These suggest that Akt plays a role in IRF3 activation as a downstream component of TRIF and TBK1.

We further determined if the modulation of IRF3 activation by Akt affects the target gene expression. Akt(DN) suppressed IFN-β–promoter reporter expression induced by poly(I:C) and LPS (Fig. 3*A*). Consistently, the protein levels of IFN-β increased by poly(I:C) and LPS were reduced by Akt(DN) (Fig. 3*B*). Akt(DN) also suppressed IFN-β–promoter reporter expression induced by TRIF and TBK1 (Fig. 3*C*). Furthermore, Akt siRNA greatly reduced poly(I:C)-induced expression of IFN-β and RANTES (Fig. 3*D*). These results show that Akt functions downstream of TRIF and TBK1 and contributes to activation of IRF3 and expression of IFN-β in the TRIF/TBK1 pathway.

Because Akt was involved in IRF3 activation, we determined if Akt interacts with IRF3. WT Akt and various forms of IRF3 were expressed in 293T cells, and coimmunoprecipitation study was performed. Akt directly interacted with constitutively active IRF3 [IRF3(5D)], but not with WT IRF3 nor inactive mutant of IRF3 [IRF3(5A)] (Fig. 4). These suggest that IRF3 may be one of the downstream targets of Akt. Moreover, the result that Akt associates preferentially with activated form of IRF3 suggests that the regulation of IRF3 by Akt may be dependent on activation signal.

TBK1 interacts with Akt

PI3K plays an important role as an upstream kinase in activation of Akt. Therefore, we tested if PI3K is involved in the TRIF pathway. LY294002 (a PI3K inhibitor) and a DN of p85 (the PI3K regulatory unit) suppressed poly(I:C)- and LPS-induced IRF3-reporter expression (Fig. 5*A*, 5*B*), but IRF3-reporter expression and IRF3 transcriptional activation induced by TRIF were not suppressed by

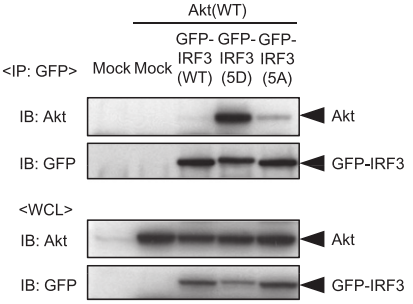

FIGURE 4. Akt associates with constitutively active IRF3. 293T cells were transfected with expression plasmids of Akt(WT), GFP-IRF3(WT), GFP-IRF3(5D), and GFP-IRF3(5A) as indicated. Cell lysates were immunoprecipitated (IP) and analyzed by immunoblotting (IB) with Abs as indicated. Expression of the proteins was confirmed by immunoblotting with whole cell lysates (WCL).

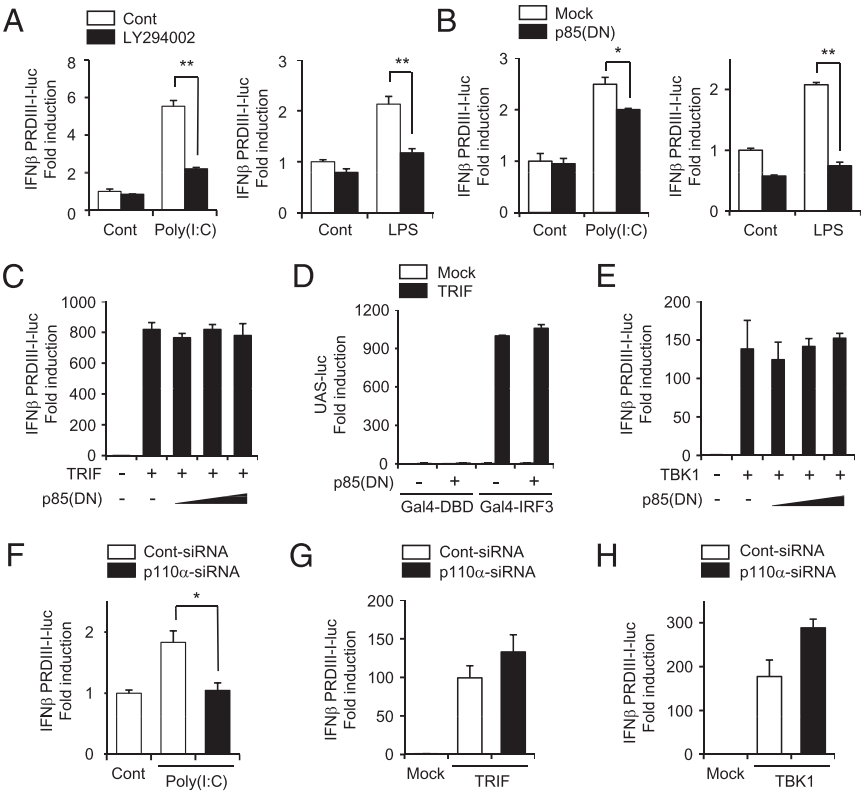

FIGURE 5. PI3K is involved in IRF3 activation induced by TLR3 and -4 agonists, but not by TRIF and TBK1. *A*, RAW264.7 cells were transfected with an IFNβ PRDIII-I-luc and stimulated with poly(I:C) (30 μg/ml) or LPS (10 ng/ml) in the presence or absence of LY294002 (20 μM) for 18 h. *B*, RAW264.7 cells were transfected with IFNβ PRDIII-I-luc and the p85(DN) expression plasmid. They were stimulated with poly(I:C) (30 μg/ml) or LPS (10 ng/ml) for 18 h. *C* and *E*, 293T cells were transfected with IFNβ PRDIII-I-luc and expression plasmids for TRIF, TBK1, and p85(DN) as indicated. *D*, 293T cells were transfected with UAS-luc and an expression plasmid for Gal4-DBD, Gal4-IRF3, TRIF, or p85 (DN). *F*, 293T cells were transfected with expression plasmids for TLR3 and IFNβ PRDIII-I-luc together with siRNA for p110α. They were further stimulated with poly(I:C) (30 μg/ml) for 18 h. *G* and *H*, 293T cells were transfected with IFNβ PRDIII-I-luc, an expression plasmid for TRIF and TBK1, and siRNA for p110α. Luciferase activities were determined and are presented as fold inductions (mean ± SEM, $n = 3$). $*p < 0.05$; $**p < 0.01$.

p85(DN) (Fig. 5*C*, 5*D*), nor was TBK1-induced IRF3-reporter expression (Fig. 5*E*). Consistent with this, siRNA for p110α (the PI3K catalytic unit) abolished poly(I:C)-induced IRF3-reporter expression, whereas p110α siRNA did not reduce TRIF- and TBK1-induced IRF3-reporter expression (Fig. 5*F–H*). These results suggest that the PI3K and Akt pathways diverge at TRIF and that Akt cooperates with TBK1 as a downstream component of the TRIF/TBK1 pathway to promote maximal activation of IRF3, whereas PI3K participates in IRF3 activation independently of TRIF and TBK1. Although PI3K is able to contribute to IRF3

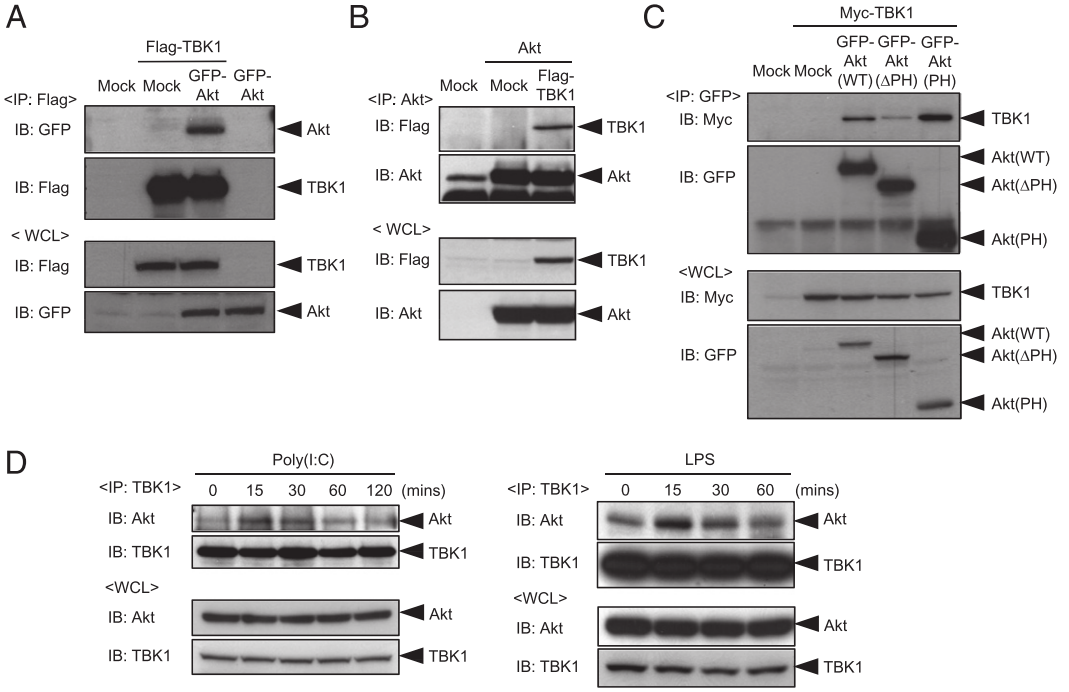

FIGURE 6. Akt associates with TBK1. 293T cells were transfected with expression plasmids of GFP-tagged Akt and flag-tagged TBK1 (*A*), Akt(WT) (non-tagged) and flag-tagged TBK1 (*B*), and GFP-tagged Akt(WT), Akt(PH), Akt(ΔPH), and myc-tagged TBK1 (*C*) as indicated. *D*, RAW264.7 cells were treated with poly(I:C) (100 μg/ml) or LPS (100 ng/ml) for the indicated times. Cell lysates were immunoprecipitated (IP) and analyzed by immunoblotting (IB) with Abs as indicated. Expression of the proteins was confirmed by immunoblotting with whole cell lysates (WCL). The results are representative of at least three separate experiments.

activation induced by TLR agonists, this may occur at the receptor level, as association of PI3K with TLR3 has been reported (17).

Because our results revealed that Akt functions as a downstream component of the TBK1 pathway, we used coimmunoprecipitation assays to test whether TBK1 interacts with Akt. TBK1 indeed associated with Akt when both were overexpressed (Fig. 6*A*, 6*B*). PH domain of Akt was preferentially associated with TBK1 (Fig. 6*C*). To demonstrate an interaction of endogenous TBK1 and Akt, RAW264.7 cells were treated with poly(I:C) and LPS for the indicated times, and cell lysates were immunoprecipitated with anti-TBK1 Ab and immunoblotted with anti-Akt Ab. The association of endogenous TBK1 and Akt was observed at 15–30 min after poly(I:C) and LPS treatment (Fig. 6*D*). Consistently, the association of endogenous Akt with exogenous TBK1 was induced in response to TLR3 and -4 agonists (Supplemental Fig. 5). After transfection of 293T cells with TLR3 or TLR4/MD2 along with flag-tagged TBK1, and stimulation with each agonist, cell lysates were then immunoprecipitated with anti-Akt Ab and

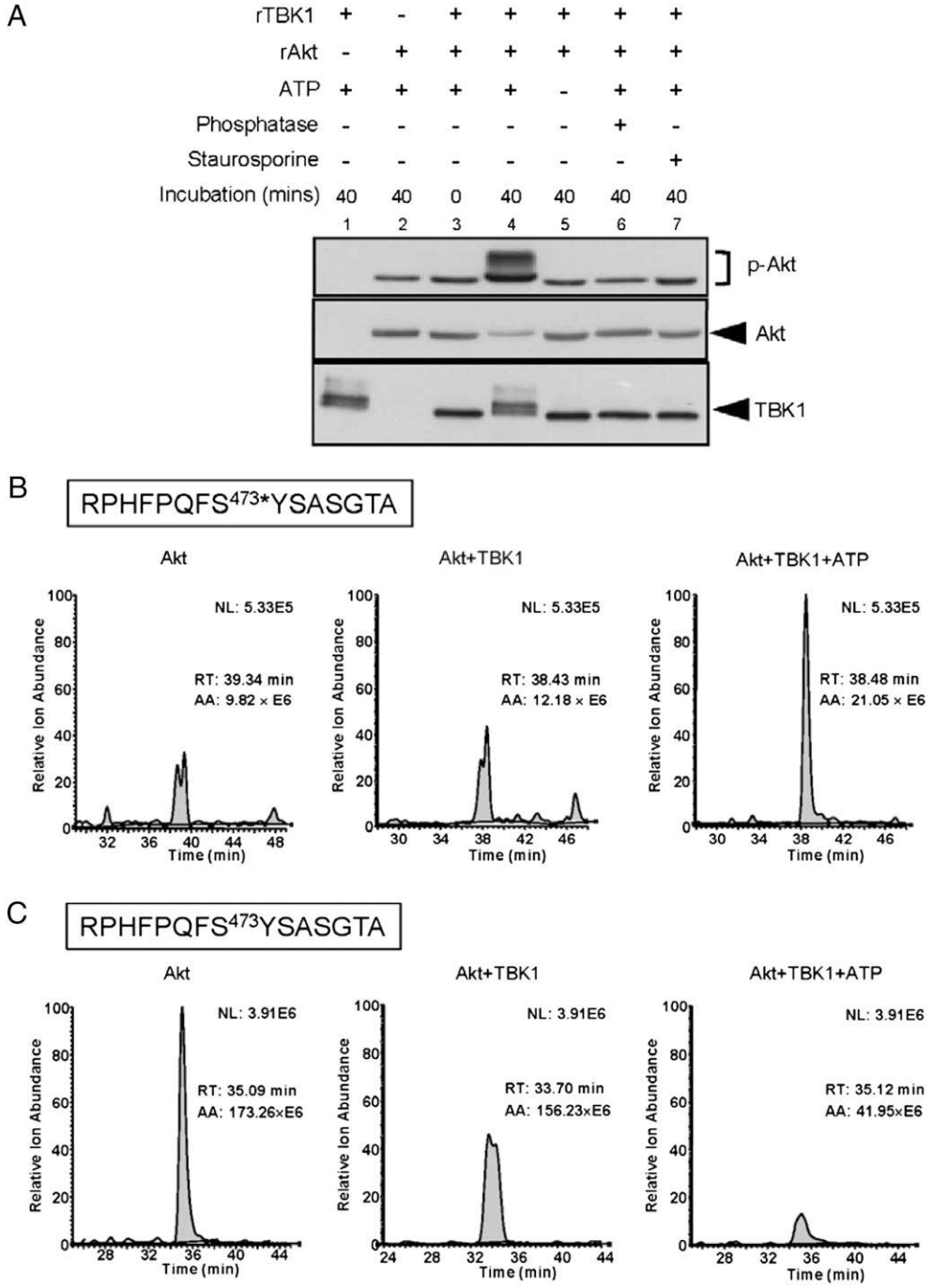

FIGURE 7. TBK1 enhances phosphorylation of Akt. *A*, In vitro TBK1 kinase assays were performed by incubating recombinant active TBK1 (rTBK1) and recombinant inactive Akt (rAkt) in the presence or absence of ATP. Phosphatase (1 U) and staurosporine (100 nM) were added to *lanes 6* and *7*, respectively. Akt activation was determined by immunoblotting for phospho(Ser^{473})-Akt. *B*, Elution profiles of a phosphorylated form of the in-gel tryptic Akt peptide, RPHFPQFS^{473}*YSASGTA, under various experimental conditions. Equal amounts of Akt in-gel tryptic digests were analyzed by RPLC-MS/MS analysis. Selected ion chromatograms of the doubly protonated form of RPHFPQFS^{473}*YSASGTA (m/z 867.19) were constructed postincubation of Akt, Akt+TBK1, and Akt+TBK1+ATP. * denotes phosphorylation site. *C*, Elution profiles of the unmodified form of RPHFPQFS^{473}YSASGTA under the same experimental conditions as *B*. Representative MS/MS spectra matching RPHFPQFS^{473}*YSASGTA and RPHFPQFS^{473}YSASGTA are presented in Supplemental Fig. 4.

immunoblotted with anti-flag Ab. Poly(I:C) and LPS caused association of endogenous Akt and flag-tagged TBK1 in a time-dependent manner. These results show that TBK1 and Akt associate with each other in response to TLR3 and -4 agonists.

TBK1 is involved in Akt phosphorylation

To examine if TBK1 directly regulates Akt activation, we performed in vitro TBK1 kinase assays by incubating recombinant active TBK1 with recombinant inactive Akt in the presence of ATP. Endogenous phosphorylation at Ser^{473}, as a readout for Akt activation, was detected with Akt alone although the level was relatively low (*lane 2* in Fig. 7*A*), but addition of TBK1 greatly enhanced Ser^{473} phosphorylation (*lane 4* in Fig. 7*A*). A distinct protein band, probably corresponding to phosphorylated Akt, was observed in this sample. Absence of ATP or addition of phosphatase or a protein kinase inhibitor (staurosporine) blocked TBK1-induced phosphorylation and formation of this band (*lanes 5–7* in Fig. 7*A*, respectively) indicating that Akt phosphorylation by TBK1 requires functional kinase activity of TBK1. To confirm phosphorylation of Akt by TBK1 and to locate the phosphorylation sites of Akt, we performed RPLC-MS/MS analyses. Selected ion chromatograms for Akt tryptic digest peptides were constructed to compare the level of phosphorylation on the identified phosphorylation sites under various experimental conditions. Low-level endogenous Ser^{473} phosphorylation was again detected in the Akt-alone sample. Ser^{473} phosphorylation was enhanced by TBK1 (Fig. 7*B*), and the proportion of unphosphorylated Ser^{473} was significantly decreased (Fig. 7*C*). Comparisons of the integrated peak areas of phosphopeptides and unmodified peptides are presented in Table I. RPLC-MS/MS analyses also revealed other sites of Akt phosphorylation, at Thr^{195} and Ser^{378}, although these residues were not as efficiently phosphorylated as Ser^{473} (data not shown). Multiple phosphorylations may account for the heterogeneous migration pattern observed in *lane 4* of Fig. 7*A*.

To investigate TBK1-dependent Akt phosphorylation in cell systems, we transfected 293T cells with expression plasmids of TBK1 (WT) and Akt(WT) and determined the level of phospho-Akt. When TBK1 was coexpressed with Akt, the level of phospho-Akt was greatly enhanced as compared with autonomous phosphorylation of Akt induced by transfection of Akt alone (*lane 3* versus *lane 4* in Fig. 8*A*). Next, we investigated if TBK1 induces Akt phosphorylation in a PI3K-independent manner. To exclude the influence of PI3K, we treated the cells with a pharmacological PI3K inhibitor, LY294002. Akt phosphorylation was still strongly induced by TBK1 in the presence of LY294002 (*lane 7* in Fig. 8*A*), whereas the phosphorylation induced by Akt alone was greatly diminished by LY294002 (*lane 8* in Fig. 8*A*). These demonstrate that TBK1-mediated Akt phosphorylation occurs in a PI3K-independent manner. To further confirm that this Akt phosphorylation is dependent on TBK1 kinase activity, we compared the effects of TBK1(WT) and kinase-inactive TBK1 [TBK1 (K38A)] on Akt phosphorylation. Although cotransfection of TBK1(WT) enhanced Akt phosphorylation, cotransfection of TBK1(K38A) did not potentiate the phosphorylation of Akt (*lane 3* versus *lane 4* in Fig. 8*B*). Consistent with the results in Fig. 7, these show that TBK1-induced Akt phosphorylation is dependent on functional kinase activity of TBK1. Interestingly, cotransfection of TBK1(K38A) rather decreased the level of phospho-Akt as compared with that increased by Akt(WT) alone (*lane 4* versus *lane 2* in Fig. 8*B*). This suggests the possibility that endogenous TBK1 activity may contribute to autonomous phosphorylation of Akt. Further addition of LY294002 together with TBK1(K38A) almost completely abolished autonomous phosphorylation of Akt induced by Akt(WT) alone (*lane 5* versus *lane 2* in Fig. 8*B*).

Table I. *Comparisons of integrated peak areas ($\times 10^6$) of phosphopeptides and unmodified peptides*

	Akt	Akt + TBK1	Akt + TBK1 + ATP
RPHFPQFS^{473}*YSASGTA	9.82	12.18	21.05
RPHFPQFS^{473}YSASGTA	173.26	156.23	41.95
Peak area ratio of phosphopeptide/unmodified peptide (%)	5.7	7.8	50.2

RPLC-MS/MS experiments were carried out using LTQ MS (Thermo Finnegan). Selected ion chromatograms were generated using a built-in feature of XCalibur software (Thermo Finnegan).

*Phosphorylation site.

These suggest the contribution and requirement of both TBK1 and PI3K pathways for the full activation of Akt. Together with results of Fig. 7, these demonstrate that TBK1 induces phosphorylation of Akt in a PI3K-independent, but TBK1 kinase activity-dependent manner.

To investigate if regulation of Akt by TBK1 is observed in cells stimulated with TLR agonist, we determined phospho-Akt in TBK1 immunoprecipitates after macrophages (RAW264.7) were stimulated with LPS. Cell lysates were immunoprecipitated with anti-TBK1 Ab and immunoblotted for phospho-Akt and TBK1. The level of phospho-Akt was enhanced in TBK1 immunoprecipitates obtained after 15 min of LPS treatment (Fig. 9*A*). This result shows that TBK1 phosphorylates Akt in response to ligand stimulation.

Next, we determined if TBK1 knockdown or knockout affects Akt phosphorylation induced by TLR3 and -4 agonists. Knockdown of TBK1 by siRNA indeed attenuated poly(I:C)- and LPS-induced Ser^{473} phosphorylation of Akt in RAW264.7 cells (Fig. 9*B*). Consistent with the results in Fig. 8*B*, the combination of TBK1 siRNA and LY294002 further reduced the level of phospho-Akt induced by LPS as compared with TBK1 siRNA or LY294002 alone (Fig. 9*C*). Furthermore, Ser^{473} phosphorylation of Akt in response to LPS or poly(I:C) was impaired in TBK1 knockout mouse embryonic fibroblasts (MEFs) (Fig. 9*D*). Whereas LPS treatment increased Akt phosphorylation in $Tbk1^{+/+}$ MEFs compared with $Tbk1^{+/+}$ control, LPS was not able to enhance Akt phosphorylation in $Tbk1^{-/-}$ MEFs compared with $Tbk1^{-/-}$ control. Similarly, Akt phosphorylation was increased by LPS and poly(I:C) in $Tbk1^{+/-}$ MEFs compared with the corresponding control in $Tbk1^{+/-}$ MEFs, whereas LPS and poly(I:C) treatment was not able to enhance Akt phosphorylation in $Tbk1^{-/-}$ MEFs compared with the control in $Tbk1^{-/-}$ MEFs. These results show that the deprivation of TBK1 rendered the cells less responsive to TLR3 or -4 agonists to induce Akt phosphorylation.

Collectively, our findings reveal that TBK1 regulates Akt phosphorylation induced by TLR3 and -4 agonists and that cooperation between these two kinases contributes to the activation of IRF3 and IFN-β expression in the TRIF-dependent signaling pathways evoked by TLR3 and -4.

Discussion

We have demonstrated a role of Akt in the regulation of IRF3 activation in the TRIF-pathway initiated by TLR3 and -4. We showed that Akt is a downstream component of the TRIF/TBK1 pathway and that TBK1 is involved in the activation of Akt induced by TLR3 and -4 agonists. The demonstration of this interaction between TBK1 and Akt adds to our understanding of innate immune signaling. Akt is a Ser/Thr kinase known to play important roles in a variety of cellular responses and in the pathogenesis of many metabolic diseases and cancers. It is activated by infection of a number of viruses, including SV40, Ebola, influenza, Sendai, and human

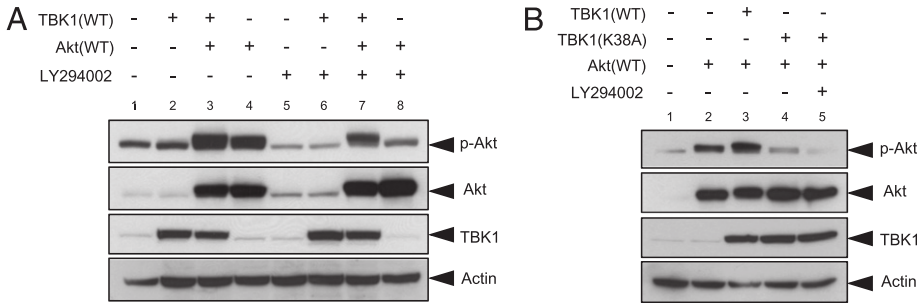

FIGURE 8. Phosphorylation of Akt by TBK1 is PI3K independent, but TBK1 kinase is activity dependent. *A* and *B*, 293T cells were transfected with expression plasmids for TBK1(WT), TBK1(K38A), and Akt(WT) as indicated in the absence or presence of LY294002 (20 μM) for 24 h. Cell lysates were analyzed for phospho (Ser^{473})-Akt, Akt, TBK1, and actin by immunoblotting.

rhinovirus, as shown by a marked increase in Akt phosphorylation. There is also a recent report that vesicular stomatitis virus induces TLR4-dependent Akt phosphorylation (18). In some cases, activation of the Akt pathway by viruses is believed to be involved in their replication, because Akt controls Coxsackie virus B3 replication and virus-induced apoptosis (19). In addition, Akt activation in response to the presence of infectious agents within innate immune cells can increase cell survival and prolong the immune response (10). Our results further reveal a function of Akt as a regulator of IRF3. Suppression of Akt with a DN or with siRNA resulted in downregulation of IRF3 activation induced by poly(I:C), LPS, TRIF, and TBK1. In addition, Akt associates with a constitutively active form of IRF3, suggesting that IRF3 may be one of the downstream targets of Akt. Because IRF3 is an important transcription factor in the production of type I IFNs, which are critical antiviral factors, the activation of Akt upon viral infection may contribute to antiviral immunity by participating in activation of IRF3 in the TRIF pathway. Our observation that TBK1 and Akt are required for maximum activation of IRF3 in that pathway suggests that this cooperation is of significance in immune responses to viral infection.

Activation of Akt is dependent on phosphorylation at Thr^{308} and Ser^{473}. Although it has been established that phosphorylation of T308 is mediated by phosphatidyl inositol-dependent kinase-1 (20, 21), it seems that Ser^{473} phosphorylation involves many effecter kinases including the rictor–mammalian target of rapamycin complex, Tcl1, DNA-PKcs, and Akt itself (22–26). We showed that association of Akt and TBK1 was induced by poly(I:C) and LPS and led to Ser^{473} phosphorylation of Akt. Phosphorylation of Thr^{195} and Ser^{378}, again dependent on TBK1 and ATP, was also observed by RPLC-MS/MS analysis. The extent of Thr^{195} and Ser^{378} phosphorylation was not as great as that of Ser^{473} phosphorylation, and it remains to be determined if these phosphorylations are induced by TLR3 and -4 agonists in intact cells and what their physiological function is.

Our results suggest that TBK1 participates in cell proliferation and cell survival by regulating Akt. In fact, TBK1 has been reported to promote tumor growth and proliferation of HUVECs (27). Hasan et al. (28) reported that TRIF/TBK1 regulates phosphorylation of the cell cycle inhibitor p27^{kip1} and proteasome degradation. Because phosphorylation of p27 can be induced by Akt (29), our results suggest that TRIF/TBK1-induced phosphorylation and degradation of p27 is at least partly mediated by Akt after it is activated by TBK1. Moreover, some viral infections are linked to cancer development: infection with human papillomavirus is associated with cervical cancer (30), and hepatitis B virus is involved in liver cancer (31). Thus, the activation of Akt by the TRIF/TBK1 pathway during viral infection may help to clarify the mechanism of tumorigenesis associated with viral infection.

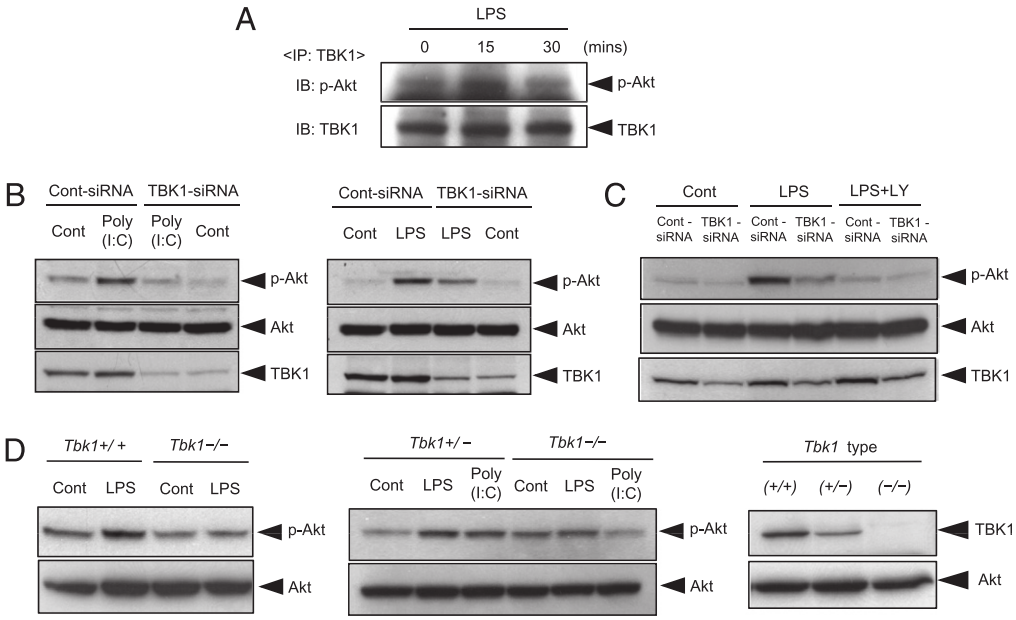

FIGURE 9. TBK1 plays a role in Akt activation induced by TLR3 and -4 agonists. *A*, After RAW264.7 cells were stimulated with LPS (100 ng/ml), cell lysates were immunoprecipitated (IP) with anti-TBK1 Ab and immunoblotted (IB) for phospho(Ser^{473})-Akt and TBK1. *B* and *C*, RAW264.7 cells were transfected with siRNA specific for TBK1 and treated with poly(I:C) (100 μg/ml) for 20 min or LPS (100 ng/ml) for 30 min in the absence or presence of LY294002 (LY, 20 μM). Cell lysates were analyzed by IB for phospho(Ser^{473})-Akt, Akt, and TBK1. *D*, WT (*Tbk1*$^{+/+}$ and *Tbk1*$^{+/-}$) and TBK1 knockout (*Tbk1*$^{-/-}$) MEFs were stimulated with poly(I:C) (100 μg/ml) or LPS (1 μg/ml) for 20 min. Cell lysates were analyzed by IB for phospho(Ser^{473})-Akt, Akt, and TBK1.

Our findings also throw light on the mechanism by which TLR signaling regulates the life span of immune cells and tumor cells by participating in apoptosis and cell proliferation.

PI3K and Akt appear to function differently in TLR signaling depending on the TLR isotype and adaptor molecules. PI3K is involved in TLR4-mediated NF-κB activation through the interaction with MyD88 (11, 32). It is also required for activation and phosphorylation of IRF3 via a direct association with TLR3, independent of TRIF (17). In addition, an inhibitor of PI3K and active PI3K did not affect IRF3-dependent promoter activity induced by TRIF, although PI3K can associate with TRIF (33). In agreement with this, we showed that p85(DN) and siRNA for p110 did not inhibit TRIF- or TBK1-induced IRF3 activation. Although PI3K appears to be required for full activation of IRF3 in TLR3 signaling via a direct interaction with TLR3 receptor, overexpression of TRIF and TBK1 is sufficient to induce IRF3 activation in the absence of PI3K (17). These observations suggest that PI3K is not involved downstream of TRIF and further that TRIF may directly activate a downstream kinase in the PI3K pathway. Our results also show that TRIF and TBK1 control the activation of Akt, which in turn contributes to activation of IRF3. The results that the simultaneous blockade of TBK1 and PI3K led to almost complete reduction of Akt phosphorylation suggest that both pathways are required for the maximal activation of Akt with a cooperative mode. It seems therefore that the PI3K and TRIF pathways converge via Akt on activation of TLR3 and -4. Akt thus plays an important role in orchestrating the PI3K and TRIF pathways in TLR signaling.

Acknowledgments

We thank Young Ju Lim for technical assistance and Dr. Julian Gross at University of Oxford, Oxford, United Kingdom, for critical reading and scientific editing.

Disclosures

The authors have no financial conflicts of interest.

References

1. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2: 675–680.
2. Medzhitov, R., and C. Janeway, Jr. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol.* 8: 452–456.
3. O'Neill, L. A. 2006. How Toll-like receptors signal: what we know and what we don't know. *Curr. Opin. Immunol.* 18: 3–9.
4. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Mühlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* 167: 5887–5894.
5. Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* 4: 491–496.
6. Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300: 1148–1151.
7. Björkbacka, H., K. A. Fitzgerald, F. Huet, X. Li, J. A. Gregory, M. A. Lee, C. M. Ordija, N. E. Dowley, D. T. Golenbock, and M. W. Freeman. 2004. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-independent signaling cascades. *Physiol. Genomics* 19: 319–330.
8. Bellacosa, A., T. O. Chan, N. N. Ahmed, K. Datta, S. Malstrom, D. Stokoe, F. McCormick, J. Feng, and P. Tsichlis. 1998. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* 17: 313–325.
9. Du, K., and P. N. Tsichlis. 2005. Regulation of the Akt kinase by interacting proteins. *Oncogene* 24: 7401–7409.
10. Park, D., N. Lapteva, M. Seethammagari, K. M. Slawin, and D. M. Spencer. 2006. An essential role for Akt1 in dendritic cell function and tumor immunotherapy. *Nat. Biotechnol.* 24: 1581–1590.
11. Lee, J. Y., J. Ye, Z. Gao, H. S. Youn, W. H. Lee, L. Zhao, N. Sizemore, and D. H. Hwang. 2003. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J. Biol. Chem.* 278: 37041–37051.
12. Lee, J. Y., L. Zhao, H. S. Youn, A. R. Weatherill, R. Tapping, L. Feng, W. H. Lee, K. A. Fitzgerald, and D. H. Hwang. 2004. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. *J. Biol. Chem.* 279: 16971–16979.
13. Ishii, K. J., T. Kawagoe, S. Koyama, K. Matsui, H. Kumar, T. Kawai, S. Uematsu, O. Takeuchi, F. Takeshita, C. Coban, and S. Akira. 2008. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 451: 725–729.
14. Stratford, S., K. L. Hoehn, F. Liu, and S. A. Summers. 2004. Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. *J. Biol. Chem.* 279: 36608–36615.
15. Youn, H. S., J. Y. Lee, K. A. Fitzgerald, H. A. Young, S. Akira, and D. H. Hwang. 2005. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. *J. Immunol.* 175: 3339–3346.
16. Yeo, M. G., B. H. Sung, H. J. Oh, Z. Y. Park, E. E. Marcantonio, and W. K. Song. 2008. Focal adhesion targeting of v-Crk is essential for FAK phosphorylation and cell migration in mouse embryo fibroblasts deficient src family kinases or p130CAS. *J. Cell. Physiol.* 214: 604–613.
17. Sarkar, S. N., K. L. Peters, C. P. Elco, S. Sakamoto, S. Pal, and G. C. Sen. 2004. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat. Struct. Mol. Biol.* 11: 1060–1067.
18. Schabbauer, G., J. Luyendyk, K. Crozat, Z. Jiang, N. Mackman, S. Bahram, and P. Georgel. 2008. TLR4/CD14-mediated PI3K activation is an essential component of interferon-dependent VSV resistance in macrophages. *Mol. Immunol.* 45: 2790–2796.
19. Esfandiarei, M., H. Luo, B. Yanagawa, A. Suarez, D. Dabiri, J. Zhang, and B. M. McManus. 2004. Protein kinase B/Akt regulates coxsackievirus B3 replication through a mechanism which is not caspase dependent. *J. Virol.* 78: 4289–4298.
20. Alessi, D. R., S. R. James, C. P. Downes, A. B. Holmes, P. R. Gaffney, C. B. Reese, and P. Cohen. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* 7: 261–269.
21. Stephens, L., K. Anderson, D. Stokoe, H. Erdjument-Bromage, G. F. Painter, A. B. Holmes, P. R. Gaffney, C. B. Reese, F. McCormick, P. Tempst, et al. 1998. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279: 710–714.
22. Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098–1101.
23. Laine, J., G. Künstle, T. Obata, M. Sha, and M. Noguchi. 2000. The protooncogene TCL1 is an Akt kinase coactivator. *Mol. Cell* 6: 395–407.
24. Pekarsky, Y., A. Koval, C. Hallas, R. Bichi, M. Tresini, S. Malstrom, G. Russo, P. Tsichlis, and C. M. Croce. 2000. Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. *Proc. Natl. Acad. Sci. USA* 97: 3028–3033.
25. Dragoi, A. M., X. Fu, S. Ivanov, P. Zhang, L. Sheng, D. Wu, G. C. Li, and W. M. Chu. 2005. DNA-PKcs, but not TLR9, is required for activation of Akt by CpG-DNA. *EMBO J.* 24: 779–789.
26. Toker, A., and A. C. Newton. 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J. Biol. Chem.* 275: 8271–8274.
27. Korherr, C., H. Gille, R. Schäfer, K. Koenig-Hoffmann, J. Dixelius, K. A. Egland, I. Pastan, and U. Brinkmann. 2006. Identification of proangiogenic genes and pathways by high-throughput functional genomics: TBK1 and the IRF3 pathway. *Proc. Natl. Acad. Sci. USA* 103: 4240–4245.
28. Hasan, U. A., C. Caux, I. Perrot, A. C. Doffin, C. Menetrier-Caux, G. Trinchieri, M. Tommasino, and J. Vlach. 2007. Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. *Proc. Natl. Acad. Sci. USA* 104: 8047–8052.
29. Liang, J., J. Zubovitz, T. Petrocelli, R. Kotchetkov, M. K. Connor, K. Han, J. H. Lee, S. Ciarallo, C. Catzavelos, R. Beniston, et al. 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat. Med.* 8: 1153–1160.
30. Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Muñoz. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 189: 12–19.
31. Kim, C. M., K. Koike, I. Saito, T. Miyamura, and G. Jay. 1991. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* 351: 317–320.
32. Ojaniemi, M., V. Glumoff, K. Harju, M. Liljeroos, K. Vuori, and M. Hallman. 2003. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur. J. Immunol.* 33: 597–605.
33. Aksoy, E., W. Vanden Berghe, S. Detienne, Z. Amraoui, K. A. Fitzgerald, G. Haegeman, M. Goldman, and F. Willems. 2005. Inhibition of phosphoinositide 3-kinase enhances TRIF-dependent NF-kappa B activation and IFN-beta synthesis downstream of Toll-like receptor 3 and 4. *Eur. J. Immunol.* 35: 2200–2209.