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Negative Regulation of Toll-like Receptor-mediated Signaling by Tollip*

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Guolong Zhang and Sankar Ghosh‡

From the Section of Immunobiology and the Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06520

Toll-like receptor (TLR)-mediated recognition of pathogens represents one of the most important mechanisms of innate immunity and disease resistance. The adaptor protein Tollip was identified initially as an intermediate in interleukin (IL)-1 signaling. Here we report that Tollip also associates directly with TLR2 and TLR4 and plays an inhibitory role in TLR-mediated cell activation. Inhibition by Tollip is mediated through its ability to potently suppress the activity of IL-1 receptor-associated kinase (IRAK) after TLR activation. In addition, we show for the first time that Tollip is a *bona fide* substrate for IRAK and is phosphorylated by IRAK upon stimulation with lipopolysaccharide or IL-1. Negative regulation of TLR signaling by Tollip may therefore serve to limit the production of proinflammatory mediators during inflammation and infection.

Toll-like receptors (TLRs)[1] represent a family of phylogenetically conserved proteins that have been found in insects, plants, and mammals (1–3). In addition to playing a critical role in the dorso-ventral axis formation in insects, TLRs are also involved in innate immunity and disease resistance (2–4). In mammals, 10 members of the TLR family have been identified which are expressed by host immune cells most likely to come into direct contact with pathogens from the environment, such as dendritic cells, peripheral phagocytes, and mucosal epithelia (5). Recent studies have revealed that a striking feature of TLRs is their ability to discriminate among different classes of pathogens (3, 4). For example, TLR4 detects lipopolysaccharide (LPS) from Gram-negative bacteria, whereas TLR2 recognizes peptidoglycan (PGN), lipoproteins, and zymosan from Gram-positive bacteria and yeast. TLRs alone or in combination are believed to mediate host immune responses to a large array of pathogens (6). In addition to detecting exogenous stimuli, TLRs are also able to trigger cell activation in response to endogenous signals, such as heat shock protein 60 (7), fibronectin (8), fibrinogen (9), and unknown factors from the injured myocardium (10) and necrotic cells (11). Activation of TLRs by endogenous ligands presumably provides immune surveillance at sites of inflammation, but sustained activation may lead to development of chronic inflammatory disorders and autoimmune diseases.

All mammalian TLRs are type I transmembrane proteins consisting of multiple copies of leucine-rich repeats in the extracellular domain and a conserved Toll/interleukin-1 receptor (IL-1R) homology (TIR) domain in the cytoplasmic tail (1, 3). Because TIR domains of TLRs, IL-1R, and IL-18R are highly homologous, it is believed that these receptors activate similar signaling pathways when stimulated with their cognate ligands (1). Consistent with this hypothesis, the downstream signaling components, myeloid differentiation protein 88 (MyD88), IL-1R-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6) are all utilized by these three groups of receptors to activate both nuclear factor (NF)-κB and mitogen-activated protein kinase signaling cascades (12–16). MyD88 functions as a crucial adaptor that links the receptors to IRAK after activation. IRAK in turn is phosphorylated, dissociates from the receptor, and associates with TRAF6 to signal activation of either NF-κB or mitogen-activated protein kinases. However, genetic studies revealed that neither MyD88 (17), IRAK (18), nor TRAF6 (19) is absolutely essential for cell activation in response to stimulation with LPS, implying the existence of alternative signaling pathways. The recent description of TIR domain-containing adaptor protein (TIRAP) (20), also known as Mal (MyD88 adaptor-like) (21), probably explains the residual signaling observed in the absence of MyD88. Interestingly TIRAP/Mal also appears to be involved in signaling through TLR4 but not IL-1, thereby demonstrating a unique specificity in the adaptor molecules that participate in signaling.

In addition to MyD88, another adaptor protein named Toll-interacting protein (Tollip) was recently found to associate with the cytoplasmic TIR domain of IL-1Rs after IL-1 stimulation (22). Tollip forms a complex with IRAK in resting cells and inhibits IL-1-induced signaling by blocking IRAK phosphorylation. Because of the significant homology in the intracellular portion of TLRs, IL-1R, and IL-18R, we hypothesized that Tollip might also inhibit TLR-mediated signaling by interacting with TLRs through the TIR domain. Here we report that Tollip associates directly with TLR2 and TLR4 and inhibits TLR-mediated cellular responses by suppressing phosphorylation and kinase activity of IRAK. Furthermore, we find that Tollip is phosphorylated by activated IRAK, making it the first direct substrate for IRAK to be characterized.

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‡ To whom correspondence should be addressed: Section of Immunobiology, Yale University School of Medicine, 310 Cedar St., New Haven, CT 06520. Tel.: 203-737-4419; Fax: 203-737-1764; E-mail: sankar.ghosh@yale.edu.

[1] The abbreviations used are: TLR(s), Toll-like receptor(s); AP-1, activator protein-1; GST, glutathione *S*-transferase; HA, hemagglutinin; IL-1R, interleukin-1 receptor; IL-18R, interleukin-18 receptor; IRAK, IL-1R-associated kinase; LPS, lipopolysaccharide; Luc, luciferase; Mal, MyD88-adaptor-like; MyD88, myeloid differentiation protein 88; NF-κB, nuclear factor-κB; PGN, peptidoglycan; TIR, Toll/IL-1R homology domain; TIRAP, TIR domain-containing adaptor protein; Tollip, Toll-interacting protein; TRAF6, tumor necrosis factor receptor-associated factor 6; UBA, ubiquitin-associated.

MATERIALS AND METHODS

Cells and Reagents—Human embryonic kidney epithelial 293 cells stably expressing the NF-κB-dependent luciferase reporter pBIIX-Luc (293-Luc) were developed as described previously (23). Mouse macrophage RAW264.7 cells were obtained from ATCC (Manassas, VA). Both cell lines were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 7% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. LPS from *Escherichia coli* 0111:B4, PGN from *Staphylococcus aureus*, anti-FLAG M2 antibody, and M2 agarose beads were purchased from Sigma. Purified polyclonal rabbit antibodies against IRAK and hemagglutinin (HA) epitope were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A-Sepharose and glutathione-conjugated agarose beads were from Amersham Biosciences, Inc.

Plasmids—Mammalian expression vectors containing N-terminal FLAG-tagged human TLR2 and TLR4 that were made by inserting PCR-generated cDNA fragments lacking the signal sequence into pFLAG-CMV-1 (Sigma) were kindly provided by R. Medzhitov (Yale Medical School). The intracellular domains of TLR2 and TLR4 were amplified by PCR and cloned into pGBKT7 (Clontech, Palo Alto, CA). Plasmids for human CD14, FLAG-tagged human Tollip, HA-tagged Tollip, and its deletion mutants were constructed in-frame by inserting PCR-generated cDNA fragments into pCDNA3.1 (Invitrogen). FLAG-IRAK and HA-IRAK were generated by inserting cDNA into pCDNA3.1. Kinase-inactive FLAG-IRAK (K239A) and HA-IRAK (K239A) were made using the Quick site-directed mutagenesis kit from Stratagene (La Jolla, CA). All FLAG and HA epitope tags were generated by incorporating tag sequences into primers at the N terminus of respective cDNA fragments. Correct inserts were confirmed by direct sequencing. The expression vector pEFBOS containing human MD-2 was kindly provided by K. Miyake (Saga Medical School, Japan).

Development of 293/TLR2 and 293/TLR4 Stable Cell Lines—293-Luc cells were seeded into 10-cm dishes and transfected using FuGene 6 (Roche Molecular Biochemicals) with 5 μg of pFLAG-TLR2 or pFLAG-TLR4 together with 0.5 μg of a plasmid encoding CD14 containing a hygromycin resistance gene. Cells were selected in Dulbecco's modified Eagle's medium with 200 μg/ml hygromycin B (Calbiochem). Individual colonies were picked, expanded, and confirmed for the surface expression of TLRs by flow cytometry and immunoblotting using anti-FLAG M2. Stable cell lines were propagated and maintained in complete Dulbecco's modified Eagle's medium for regular 293-Luc cells.

Transfection and Luciferase Reporter Assays—RAW264.7 cells were plated at a density of 3×10^5 cells/well in 24-well plates 1 day before transfection. Cells were transfected in triplicate using FuGene 6 with 0.2 μg of pBIIX-Luc or pAP-1-Luc and the indicated amounts of HA-Tollip plasmid. The total amount of transfected plasmids was equalized by supplementing with the empty vector pCDNA3.1. After 24 h, cells were either left untreated or stimulated with 100 ng/ml LPS or 10 μg/ml PGN for 6 h. Cells were then lysed and assayed for luciferase activity (Promega, Madison, WI). The data were normalized to total protein concentrations of each sample measured using a protein assay kit from Bio-Rad.

Immunoprecipitation and Immunoblotting—For coimmunoprecipitation of transfected proteins, 7×10^5 293, 293/TLR2, or 293/TLR4 cells were plated into six-well plates and transfected the following day with the indicated plasmids using FuGene 6. 24–36 h after transfection, cells were lysed in 0.5 ml of TNT buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 1% Triton X-100) containing a protease inhibitor mixture (Roche Molecular Biochemicals). A portion of the lysates (50 μl) was saved for immunoblotting, and FLAG-tagged proteins were precipitated from the remainder for 2–4 h at 4 °C with 20 μl of 50% (v/v) slurry of anti-FLAG M2 beads. After extensive washing with TNT buffer, the beads were boiled in SDS sample buffer. Proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with the indicated antibodies. The reactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.).

Generation of Glutathione S-Transferase (GST)-Tollip and in Vitro Pull-down Assays—GST-Tollip and its N-terminal (1–52), central (53–178), and C-terminal (179–273) truncation mutants were made by cloning respective human Tollip cDNA fragments in-frame into pGEX-4T-1 (Amersham Biosciences, Inc.). GST fusion proteins were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 4–6 h at 37 °C and incubated with glutathione beads. Purified GST proteins were obtained by elution from the beads with 10 mM reduced glutathione followed by dialysis in phosphate-buffered saline. [^{35}S]Methionine-labeled proteins were generated with TNT-T7 Quick-coupled transcription/translation system (Promega) according to the manufacturer's instructions. *In vitro* GST pull-down assays were performed essentially as described previously (24). Briefly, 5 μl of ^{35}S-labeled lysates was incubated for 1 h at 4 °C with 5 μg of purified GST or GST-Tollip and 20 μl of glutathione beads in 1 ml of TNT buffer. The beads were washed three times with TNT buffer, boiled in SDS sample buffer, and fractionated by 12.5% SDS-PAGE. The radiolabeled proteins were visualized by autoradiography.

In Vitro Kinase Assays—For the detection of endogenous IRAK activity, 3×10^6 293/TLR2 or 293/TLR4 cells were plated into 10-cm dishes and transfected the following day with 5 μg of HA-Tollip and/or 5 μg of MD-2 plasmid using FuGene 6. 24 h after transfection, cells were either left untreated or stimulated with 1 μg/ml LPS for 10 or 30 min and lysed in 1.2 ml of TNT buffer containing 2 mM NaF, 1 mM dithiothreitol, 1 mM EDTA, and a mixture of protease inhibitors. Cell lysates were precipitated at 4 °C for 4 h with 1 μg of anti-IRAK antibody and 20 μl of 50% (v/v) slurry of protein A-Sepharose beads. IRAK autophosphorylation was measured essentially as described previously using protocols developed for detecting IKK autophosphorylation (24). After washing twice with TNT buffer and twice with kinase buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM NaF, 1 mM dithiothreitol, 10 μM ATP), the beads were suspended in 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (Amersham Biosciences, Inc.). The reactions were allowed to proceed at 30 °C for 20 min and terminated with 20 μl of SDS sample buffer. The beads were boiled, 20-μl reaction aliquots were separated by 10% SDS-PAGE, and visualized by autoradiography. For measuring phosphorylation of Tollip by IRAK, IRAK was first precipitated, and immune complexes were washed with TNT and kinase buffers, then incubated in 25 μl of kinase buffer with 10 μCi of [γ-^{32}P]ATP and 2 μg of purified GST, GST-Tollip, or its truncation mutants. Phosphorylated proteins were precipitated further with 20 μl of glutathione-agarose beads, separated by SDS-PAGE, and visualized by autoradiography.

RESULTS

Tollip Physically Associates with TLR2 and TLR4 via Its C-Terminal Domain—To determine whether Tollip interacts with TLRs via the TIR domain, we developed two cell lines (293/TLR2 and 293/TLR4) that stably express human CD14, as well as FLAG-tagged human TLR2 or TLR4. Flow cytometry and immunoblotting revealed significant amounts of TLR expression on the cell surface (data not shown). Furthermore, expression of the TLRs rendered the 293 cells highly responsive to various bacterial products, which do not otherwise activate these cells (data not shown).

Interactions between Tollip and TLRs were examined by transfecting these cell lines with plasmids expressing HA-Tollip and/or MD-2, which is a secreted molecule that binds to TLR4 and is required for signaling (25). The FLAG-tagged TLRs were immunoprecipitated using anti-FLAG antibody followed by immunoblotting with anti-HA antibody. HA-Tollip coprecipitated efficiently with TLR2 (Fig. 1*A*, *lane 3* of the *top panel*). Association between Tollip and TLR4 was also detectable but was enhanced significantly in the presence of MD-2 (Fig. 1*A*, *lanes 5* and *6* of the *top panel*), suggesting that conformational changes in the cytoplasmic tail of TLR4 induced by MD-2 may be necessary for efficient binding to Tollip. To determine whether Tollip directly interacts with the TIR domain of TLRs, *in vitro* GST pull-down assays were performed. [^{35}S]Methionine-labeled intracellular portions of TLR2 and TLR4 were produced by *in vitro* translation and incubated with either GST or GST-Tollip. As shown in Fig. 1*B*, both TLR2 and TLR4 bound to GST-Tollip, but not GST alone, demonstrating that Tollip associates directly with the TIR domain of TLRs.

To map the region of Tollip responsible for association with the TLRs, we analyzed the interaction of TLR2 and TLR4 with truncation mutants of Tollip as indicated in Fig. 2*A*. TLR4 bound efficiently to either full-length Tollip (1–273) or mutants with an intact C-terminal sequence, Tollip (Δ53–178) and Tollip (53–273) (Fig. 2*B*, *lanes 1*, *4* and *5* of the *top panel*). Also, whereas Tollip (1–229) could interact with TLR4, no association was found between TLR4 and Tollip (1–178) or Tollip

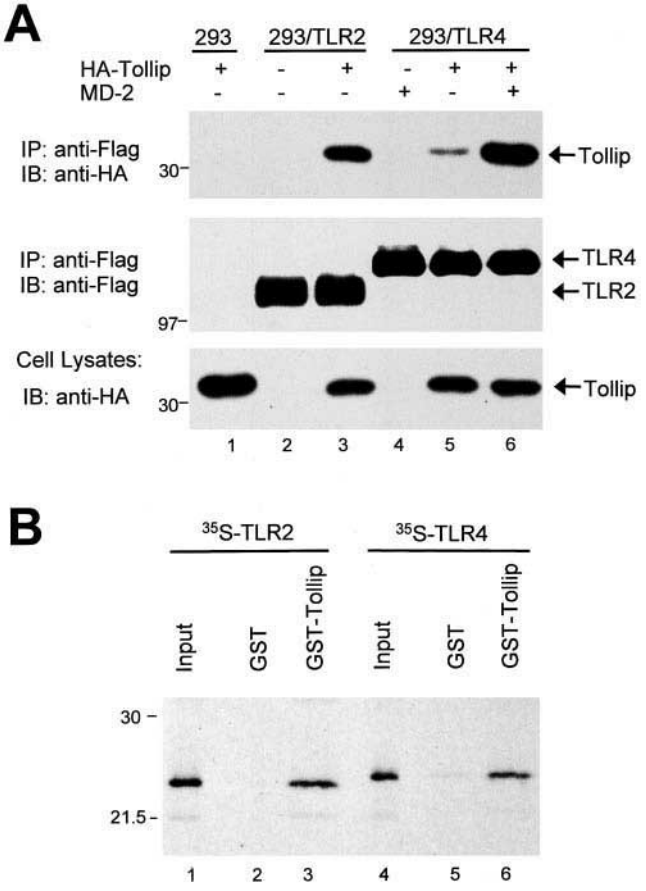

FIG. 1. **Tollip associates directly with TLR2 and TLR4.** *A*, coimmunoprecipitation of Tollip with TLR2 and TLR4. 293, 293/FLAG-TLR2, or 293/FLAG-TLR4 cells were transfected with expression plasmids as indicated (1 μg of each); after 24 h, proteins in the cell lysates were immuoprecipitated (*IP*) using anti-FLAG M2 beads. Coprecipitated proteins were detected by immunoblotting (*IB*) with anti-HA and anti-FLAG antibodies. Cell lysates (20 μl) were also immunoblotted using anti-HA antibody to monitor the expression of transfected Tollip. *B*, direct interactions of Tollip with TLR2 and TLR4. Intracellular domains of TLR2 and TLR4 were ^{35}S labeled by *in vitro* translation and incubated with purified GST or GST-Tollip bound to glutathione beads. The beads were then washed, boiled in SDS sample buffer, and fractionated by 12.5% SDS-PAGE. The radiolabeled proteins were visualized by exposure to x-ray film.

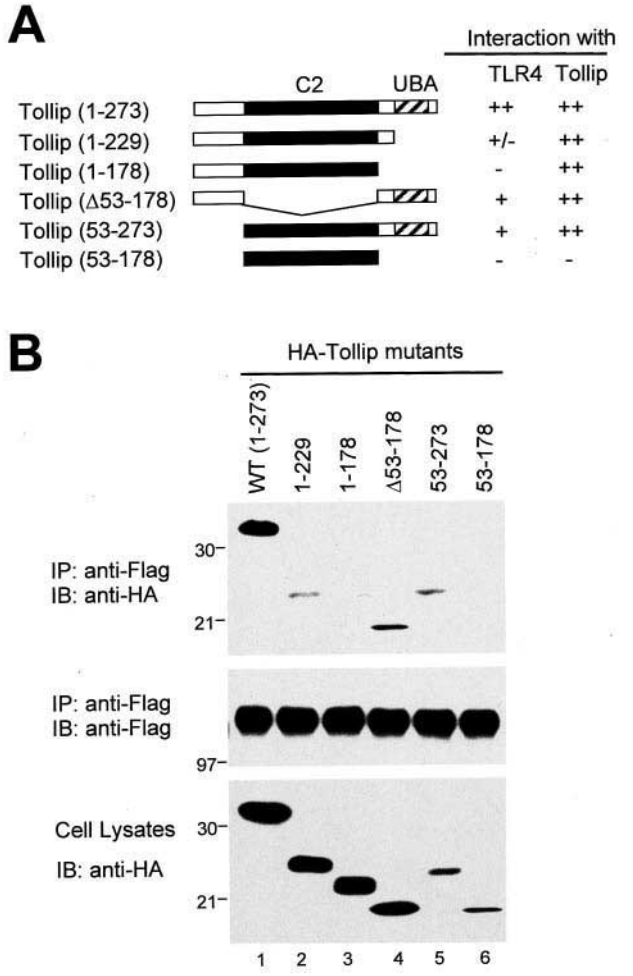

FIG. 2. **Analysis of domain interactions of Tollip with TLR4.** *A*, coimmunoprecipitation of Tollip mutants with TLR4. 293/FLAG-TLR4 cells were cultured in six-well plates and transfected with 1 μg of MD-2 plasmid together with 0.5 μg of the indicated HA-Tollip mutants. Immunoprecipitation and immunoblotting were performed as described in the legend to Fig. 1*A*. *B*, analysis of interactions of Tollip mutants with TLR4 and Tollip. Diagrammatic representations of Tollip mutants are shown; *black* segments represent the C2 domain, and *striped* segments are the UBA domain. ++, very strong; +, strong; ±, weak; −, no interaction.

(53–178), indicating that the interaction domain lies in the C-terminal region of Tollip between residues 179 and 229 (Fig. 2*B*, *lanes 2*, *3* and *6* of the *top panel*). Similar results were observed with TLR2 (data not shown). Interestingly, binding of Tollip to IL-1R is also mediated through a similar C-terminal region (22).

Tollip Interacts with Itself to Form Oligomers—TLRs, IL-1R, and IL-18R are all known to form either homo- or heterodimers upon stimulation with their cognate ligands. Therefore, it is possible that downstream proteins might also oligomerize to allow signals to be transduced efficiently. To test whether Tollip forms homo-oligomers, we *in vitro* translated full-length ^{35}S-labeled Tollip (1–273) and used it in GST pull-down assays. We found that Tollip bound to GST-Tollip but not to GST alone (Fig. 3*A*), suggesting that Tollip is capable of self-association. To identify further the domains involved in Tollip homo-oligomerization, 293-Luc cells were cotransfected with FLAG-tagged full-length Tollip and the HA-tagged Tollip deletion mutants described in Fig. 2*A*, and the interaction between the proteins was analyzed by coimmunoprecipitation (Fig. 3*B*). All Tollip mutants associated efficiently with full-length Tollip except for the construct containing only the C2 domain (53–178) (Figs. 3*B* and 2*A*). Therefore, self-association of Tollip is mediated by both the N- and C-terminal regions of the protein and may result in formation of a stable protein complex.

Although both Tollip and MyD88 function as adaptors to link IL-1R or TLRs to downstream molecules, neither *in vitro* GST pull-down nor *in vivo* mammalian coimmunoprecipitation assays revealed a stable complex between these two molecules (data not shown). These results suggested that MyD88 and Tollip exist in distinct protein complexes, which is consistent with previous findings that both molecules are recruited independently to the IL-1R after stimulation (22).

Tollip Inhibits Cell Activation Induced by Bacterial Products—To assess the functional role of Tollip in TLR-mediated signaling, we transfected mouse macrophage RAW264.7 cells with increasing amounts of Tollip together with either NF-κB or AP-1 luciferase reporter vectors. Luciferase activity was measured after stimulation of the transfected cells with various bacterial products. Both NF-κB and AP-1 were activated readily in response to LPS (Fig. 4) and PGN (data not shown), and both were inhibited dose-dependently by Tollip, suggesting that Tollip is a negative regulator of TLR2 and TLR4 signaling. Overexpression of Tollip also impaired NF-κB activation in

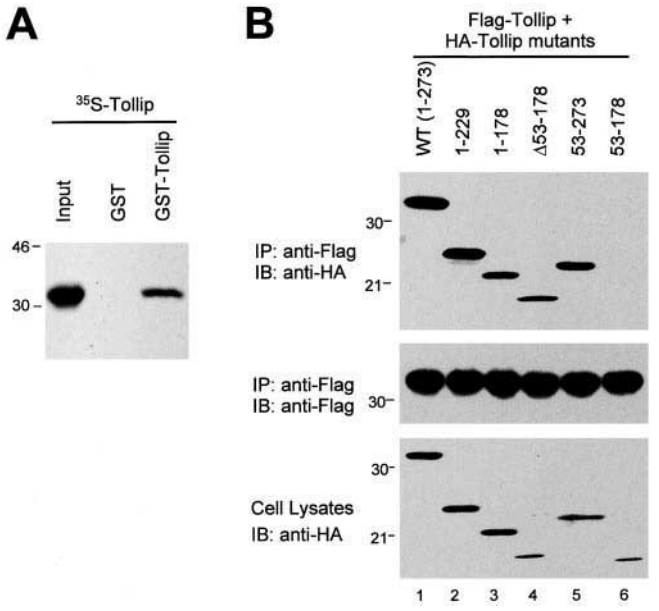

FIG. 3. **Tollip forms oligomers.** *A*, self-association of Tollip. Tollip was translated *in vitro* and incubated with purified GST or GST-Tollip bound to glutathione beads. The pull-down assay was performed as described in the legend to Fig. 1*B*. *B*, analysis of domains involved in self-association of Tollip. 293-Luc cells were cultured in six-well plates and transfected with 0.5 μg of FLAG-Tollip plasmid together with 0.5 μg of the indicated HA-Tollip deletion mutants. Immunoprecipitation and immunoblotting were performed as described in the legend to Fig. 1*A*.

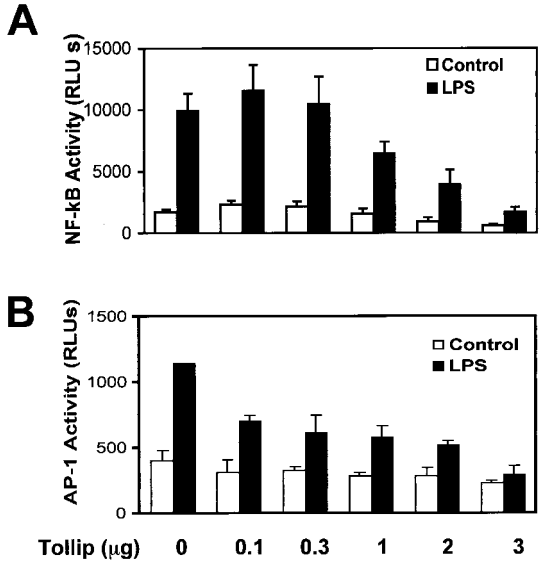

FIG. 4. **Tollip impairs LPS-induced cell activation.** RAW264.7 cells were plated in 24-well plates and transfected in triplicate with 0.2 μg of luciferase reporter vector pBIIX-Luc or pAP-1-Luc together with the indicated amounts of HA-Tollip plasmid. After 24 h, cells were either left untreated (*white bars*) or stimulated (*black bars*) with 100 ng/ml LPS for a further 6 h. Cells were then lysed and assayed for luciferase activity. The data were normalized to total protein concentrations and are represented as the means ± S.D. Similar results were obtained from three independent experiments. These data demonstrate that Tollip inhibits activation of both NF-κB (*A*) and AP-1 (*B*) in a dose-dependent manner.

293/TLR2 and 293/TLR4 cells after stimulation with LPS or PGN (data not shown).

Tollip Suppresses the Phosphorylation and Kinase Activity of IRAK—To understand better the molecular mechanism by which Tollip inhibits TLR-mediated signaling, we analyzed its effect on the recruitment of IRAK to the TLR complex. 293/

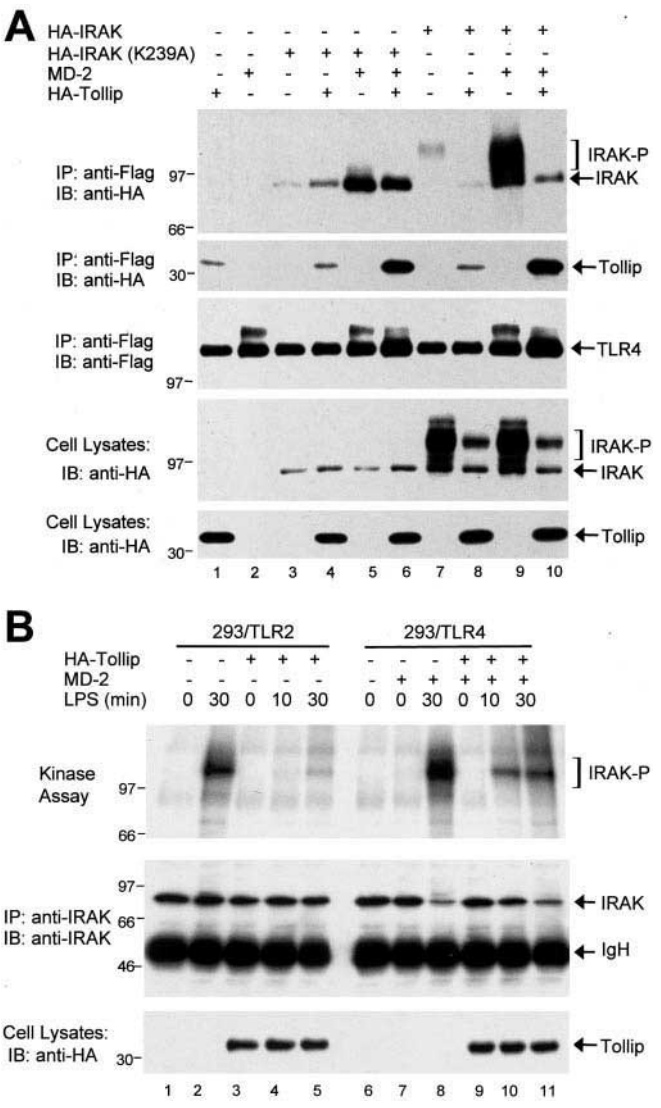

FIG. 5. **Tollip suppresses phosphorylation and kinase activity of IRAK.** *A*, inhibition of the recruitment of phosphorylated IRAK to the TLR4 receptor complex. 293/FLAG-TLR4 cells were cultured in six-well plates and transfected with 1 μg of each of the indicated plasmids. Immunoprecipitation and immunoblotting were performed as described in the legend to Fig. 1*A*. *B*, suppression of LPS-induced kinase activity of IRAK by Tollip. 293/FLAG-TLR2 and 293/FLAG-TLR4 cells were cultured in 10-cm dishes and transfected with the plasmids indicated (5 μg of each). 24 h after transfection, cells were either left untreated or stimulated with 1 μg/ml LPS for 10 or 30 min. Proteins in cell lysates were precipitated using anti-IRAK antibody and protein A-Sepharose beads. Immune complexes were washed, and an aliquot of the immunoprecipitate was saved for immunoblotting to confirm equal loading. The remainder of the precipitated proteins was incubated in kinase buffer at 30 °C for 20 min, separated by SDS-PAGE, and phosphorylated IRAK was visualized by autoradiography.

FLAG-TLR2 and 293/FLAG-TLR4 cells were transfected with Tollip together with either wild type or kinase-inactive (K239A) IRAK. Consistent with previous findings, transfection of wild type IRAK resulted in significant autophosphorylation of IRAK; however, the level of phosphorylation was diminished dramatically in the presence of Tollip (Ref. 22 and Fig. 5*A*, *lanes 7* and *9 versus 8* and *10* of the *second panel* from the *bottom*). Immunoprecipitation of TLR4 with anti-FLAG antibody revealed that Tollip did not appear to influence the amount of IRAK (K239A) recruited to the TLR4 receptor complex in the presence or absence of MD-2 (Fig. 5*A*, *lanes 3* and *4 versus 5* and *6* of the *top panel*). In contrast, recruitment of phosphorylated IRAK was completely abolished by Tollip re-

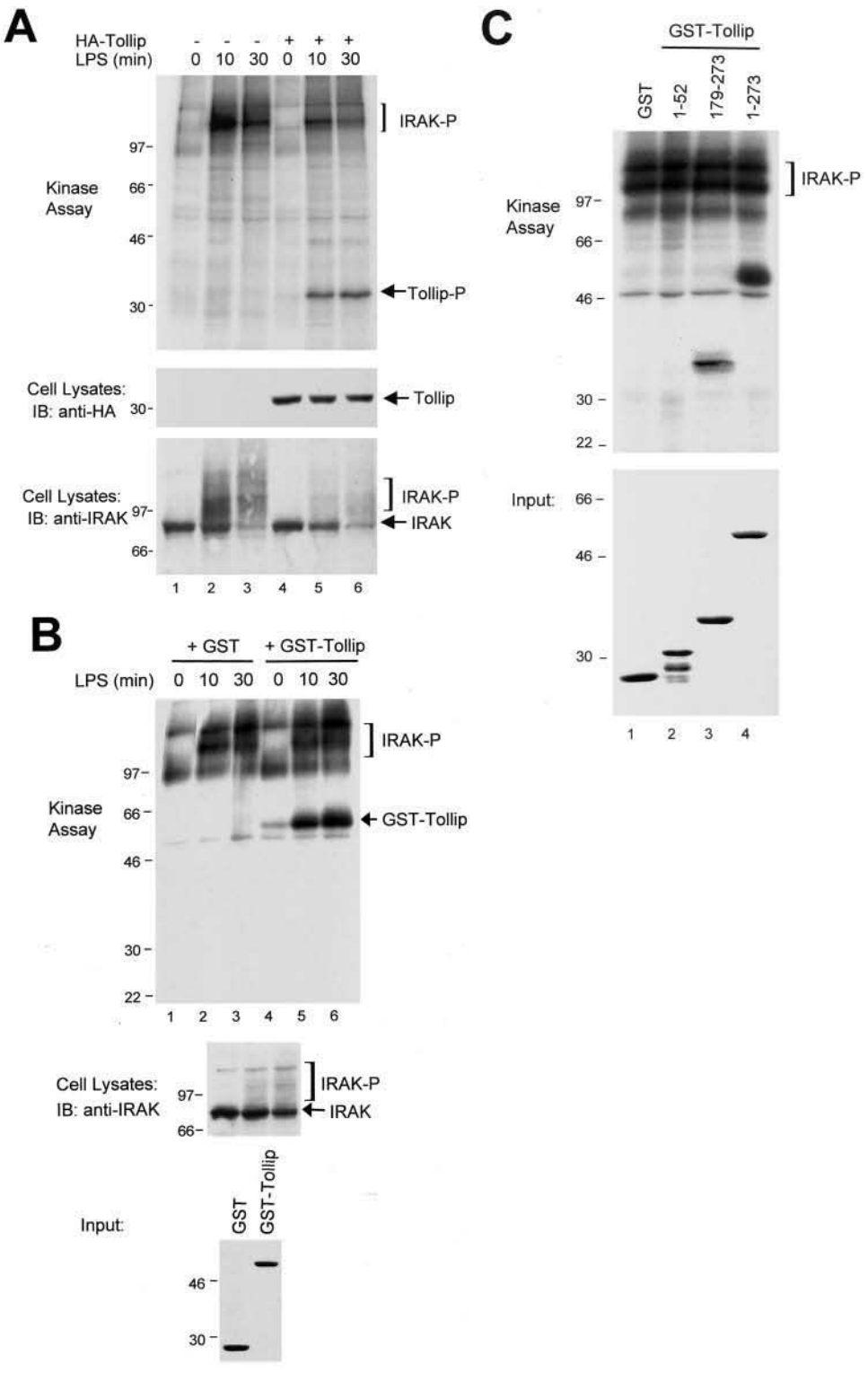

FIG. 6. **Tollip is phosphorylated by activated IRAK.** *A*, phosphorylation of Tollip by IRAK. 293/FLAG-TLR4 cells were cultured in 10-cm dishes and transfected with 5 μg of MD-2 plasmid with or without 5 μg of HA-Tollip. 24 h after transfection, cells were either left untreated or stimulated with 1 μg/ml LPS for 10–30 min. Cell lysates were precipitated with anti-IRAK antibody and protein A-Sepharose beads, and kinase assays were performed as described in the legend to Fig. 5*B*. The lysates (20 μl) were also blotted with anti-IRAK and anti-HA antibodies to monitor the expression of transfected Tollip and phosphorylation of endogenous IRAK upon stimulation. *B*, phosphorylation of GST-Tollip by IRAK. 293/FLAG-TLR4 cells were cultured in six-well plates and transfected with 1 μg/well MD-2 plasmid. Cells were then left untreated or stimulated with 1 μg/ml LPS for 10–30 min, and lysates were precipitated with anti-IRAK antibody and protein A-Sepharose beads. Kinase assays were performed in the presence of 2 μg of purified GST or GST-Tollip. Phosphorylated proteins were precipitated further with glutathione beads, separated by SDS-PAGE, and visualized by autoradiography. Approximately equal amounts of GST protein input are shown on the *bottom panel*. *C*, Tollip is phosphorylated by IRAK at the C terminus. 293/FLAG-TLR4 cells were seeded in six-well plates and transfected with 1 μg/well MD-2 plasmid. After 24 h, cells were stimulated with 1 μg/ml LPS for 20 min, and lysates were precipitated with anti-IRAK antibody and protein A-Sepharose beads. Kinase assays were performed in the presence of 2 μg of purified GST, GST-Tollip, or its truncation mutants. The *bottom panel* shows approximately equal amounts of GST proteins used in the kinase assay.

gardless of MD-2 expression (Fig. 5*A*, *lanes 7* and *9 versus 8* and *10* of the *top panel*). Similar results were observed in 293/FLAG-TLR2 cells (data not shown).

In vitro kinase assays were also performed to confirm that suppression of IRAK autophosphorylation by Tollip was the result of decreased IRAK kinase activity. 293/FLAG-TLR2 and 293/FLAG-TLR4 cells were transfected with Tollip and/or MD-2, stimulated with LPS, and then IRAK was immunoprecipitated and assayed for its kinase activity. As expected, IRAK was heavily phosphorylated after LPS stimulation (Fig. 5B, *lanes 2* and *8* of the *top panel*), but this autophosphorylation was decreased dramatically in the presence of Tollip (Fig. 5*B*, *lanes 4*, *5*, *10*, and *11* of the *top panel*). These findings suggest a possible mechanism by which Tollip might impair TLR signaling, namely by blocking the autophosphorylation of IRAK, which is necessary for the dissociation of IRAK from the receptor complex (26, 27).

Tollip Is Phosphorylated by IRAK—IRAK is a serine/threonine kinase with a centrally located catalytic domain (13). Upon stimulation with either IL-1 or bacterial products, IRAK is activated and heavily phosphorylated (Ref. 13 and Fig. 6*A*, *lanes 2* and *3* of both *top* and *bottom panels*). However, the identity of potential IRAK substrate(s) has remained unknown. In addition, recent studies have suggested that the kinase activity of IRAK is dispensable for its role as an intermediate in IL-1 signaling (28, 29). However, we were surprised to find that after LPS stimulation, activation of IRAK is accompanied by a concomitant phosphorylation of Tollip in 293/FLAG-TLR4 (Fig. 6*A*, *lanes 5* and *6* of the *top panel*) and 293/FLAG-TLR2 cells (data not shown). Phosphorylation of Tollip did not occur in resting cells without activated IRAK (Fig. 6*A*, *lane 4* of the *top panel*).

To confirm this observation, the 293/FLAG-TLR2 and 293/FLAG-TLR4 cells were either left untreated or stimulated with LPS. IRAK was immunoprecipitated from these cells and incubated with purified GST-Tollip in *in vitro* kinase assays. We found that upon stimulation with LPS, concordant with the phosphorylation of IRAK, GST-Tollip (Fig. 6*B*, *lanes 4–6* of the *top panel*), but not GST alone (Fig. 6*B*, *lanes 1–3* of the *top panel*), was heavily phosphorylated. Similar results were also observed in 293-Luc cells stimulated with IL-1 (data not shown). Therefore these experiments strongly suggest that in addition to undergoing autophosphorylation, IRAK also phosphorylates Tollip. Phosphorylation of Tollip by activated IRAK may facilitate the dissociation of IRAK from Tollip, thereby allowing IRAK to escape the inhibitory effect of Tollip and subsequently modify downstream signaling components.

To determine further the domain of Tollip which is phosphorylated by IRAK, we produced N-terminal (1–52), central (53–178), and C-terminal (179–273) truncation mutants of Tollip fused with GST. However, the central region of Tollip, which encompasses only the C2 domain, is completely insoluble when expressed in bacteria. Therefore, kinase assays were performed with purified N- and C-terminal Tollip domains together with wild type GST-Tollip. As shown in Fig. 6*C*, the C-terminal (179–273) but not N-terminal (1–52) region of Tollip is phosphorylated by IRAK. Interestingly, the same region is also responsible for the interactions of Tollip with TLRs and IRAK (Fig. 2 and Ref. 22). However, we cannot rule out the possibility that the central C2 domain of Tollip might also be phosphorylated by IRAK upon activation because the intensity of phosphorylation at the C terminus is considerably weaker than that for wild type Tollip (Fig. 6*C*, *lane 3 versus* 4 of the *top panel*).

DISCUSSION

In this paper we demonstrate that the adaptor protein Tollip can negatively regulate TLR signaling pathways. Described initially as a protein involved in IL-1 signaling, we now show that Tollip can associate with TLR2 and TLR4 and play an inhibitory role in TLR-mediated cell activation. Consistent with our findings, during the preparation of the manuscript, Bulut *et al.* (31) reported independently that Tollip coprecipitates with TLR2 and TLR4, and overexpression of Tollip inhibits TLR2- and TLR4-induced NF-κB activation in human dermal microvessel endothelial cells.

One striking feature of Tollip is its ability to suppress the kinase activity of IRAK. Several lines of evidence suggest that the mechanism by which Tollip inhibits TLR signaling is by preassociating with IRAK, thus preventing it from being phosphorylated and activated on the TLR receptor complex. In fact, studies using IRAK-deficient cells demonstrated that phosphorylation of IRAK is required for it to dissociate from the receptor and transduce signals to downstream molecules leading to NF-κB activation (26, 27). The ability of Tollip to interact directly with both the death (1–208) and kinase (181–545) domains of IRAK may explain the inhibitory role of Tollip (22, and data not shown).

Upon activation, IRAK undergoes rapid autophosphorylation and dissociates from the receptor. Our studies reveal that after stimulation IRAK also phosphorylates Tollip. The physiological significance of this phosphorylation is still unclear, although it is possible that phosphorylation of Tollip may facilitate its dissociation from IRAK and subsequent degradation by ubiquitination. This is consistent with the fact that Tollip contains a highly conserved UBA (ubiquitin-associated) or CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation) domain at the C terminus (230–270). Similar domains in other proteins have been shown to recruit ubiquitin-conjugating enzymes, leading to proteosome-dependent degradation (32, 33). Interestingly, the region around the UBA domain of Tollip binds to unphosphorylated IRAK under resting conditions and also associates transiently with the TLRs and IL-R upon stimulation (22). The same region of Tollip can also be phosphorylated by IRAK (Fig. 6*C*). Therefore, once activated on the receptor, IRAK phosphorylates Tollip, which may lead to the dissociation of Tollip from IRAK and the receptor complex. Unmasking the UBA domain on Tollip may lead to its subsequent degradation by a ubiquitination-dependent process. The removal of Tollip would allow signaling to continue by freeing activated IRAK to bind to downstream TRAF6. Because IRAK also undergoes rapid ubiquitination and degradation after activation (13, 34), it is tempting to speculate that phosphorylated Tollip facilitates the ubiquitination of IRAK by recruiting ubiquitin-conjugating enzymes. Further studies will be needed to explore fully the biological ramifications of Tollip phosphorylation by IRAK.

Although a more complete understanding of biological functions of Tollip awaits the generation and analysis of mice lacking Tollip, it is possible that the primary role of Tollip-mediated pathway may be to maintain immune cells in a quiescent state in the absence of infection and facilitate the termination of TLR-induced cell signaling during inflammation and infection. It is conceivable that this evolutionarily conserved inhibitory system benefits the host by limiting the production of proinflammatory mediators and subsequent tissue damage under infectious conditions. Therefore, strategies to up-regulate Tollip expression may prove effective in treating both chronic and acute inflammatory diseases, such as inflammatory bowel disease and septic shock.

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