THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 15, pp. 10489–10495, April 14, 2006

MyD88 Adapter-like (Mal) Is Phosphorylated by Bruton's Tyrosine Kinase during TLR2 and TLR4 Signal Transduction*

Received for publication, August 11, 2005, and in revised form, December 20, 2005 Published, JBC Papers in Press, January 26, 2006, DOI 10.1074/jbc.M508892200

Pearl Gray, Aisling Dunne, Constantinos Brikos, Caroline A. Jefferies, Sarah L. Doyle, and Luke A. J. O'Neill[1]

From the School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland

Members of the Toll-like receptor (TLR) family are essential players in activating the host innate immune response against infectious microorganisms. All TLRs signal through Toll/interleukin 1 receptor domain-containing adapter proteins. MyD88 adapter-like (Mal) is one such adapter that specifically is involved in TLR2 and TLR4 signaling. When overexpressed we have found that Mal undergoes tyrosine phosphorylation. Three possible phospho-accepting tyrosines were identified at positions 86, 106, and 187, and two mutant forms of Mal in which tyrosines 86 and 187 were mutated to phenylalanine acted as dominant negative inhibitors of NF-κB activation by lipopolysaccharide (LPS). Activation of THP-1 monocytic cells with the TLR4 agonist LPS and the TLR2 agonist macrophage-activating lipopeptide-2 induced phosphorylation of Mal on tyrosine residues. We found that the Bruton's tyrosine kinase (Btk) inhibitor LFM-A13 could block the endogenous phosphorylation of Mal on tyrosine in cells treated with macrophage-activating lipopeptide-2 or LPS. Furthermore, Btk immunoprecipitated from THP-1 cells activated by LPS could phosphorylate Mal. Our study therefore provides the first demonstration of the key role of Mal phosphorylation on tyrosine during signaling by TLR2 and TLR4 and identifies a novel function for Btk as the kinase involved.

The primary role of the Toll-like receptor (TLR)[2] family is to recognize and signal the influx of invading pathogens, thereby activating the host innate immune response. Ten TLRs have been discovered in humans, and all have a conserved cytosolic domain termed the Toll/interleukin-1 receptor (TIR) domain that mediates signaling by TLRs (1). Once the TLRs are activated by their respective agonists, receptor dimerization via the TIR domain is thought to occur. This leads to recruitment of cytosolic TIR domain-containing adapter proteins (1). Although TLRs have similar signal transduction pathways, recent evidence has emerged suggesting specificity with regard to recruitment of the adapters. The prototype TIR-containing adapter protein, MyD88, is recruited by all TLRs with the exception of the double-stranded RNA receptor, TLR3 (2). MyD88 adapter-like (Mal) participates solely in TLR2 and TLR4 signal transduction where it may act as a bridging adapter for MyD88 recruitment (3–6). Evidence has shown that the absence of either MyD88 or Mal results in no tumor necrosis factor production and delayed activation of the transcription factor NF-κB in response to the respective TLR2 and TLR4 ligands, macrophage-activating lipopeptide-2 (MALP-2) and lipopolysaccharide (LPS) (5–8). Similar to Mal, TIR domain-containing adapter-inducing interferon (TRIF) and TRIF-related adapter molecule (TRAM) are selective adapters, with TRIF mediating TLR3 and TLR4 signaling, whereas TRAM is essential for TLR4 signaling alone (9–13). It has been determined that TRIF and TRAM mediate activation of another transcription factor termed interferon regulatory factor 3 in addition to NF-κB.

Several serine/threonine protein kinases are activated during TLR signal transduction. Mal and MyD88 recruitment leads to the subsequent recruitment and activation of interleukin 1 (IL-1) receptor-associated kinase 4, which in turn activates IL-1 receptor-associated kinase 1 (14). Tumor necrosis factor receptor-associated factor 6 is then recruited, leading to activation of TGF-β-activated kinase (15). TGF-β-activated kinase then activates the IκB kinase complex, leading to NF-κB activation (16). TGF-β-activated kinase 1 can also activate the upstream kinases p38 mitogen-activated protein kinase and Jun-N-terminal kinase. TRAM and TRIF recruitment leads to activation of the kinase receptor-interacting protein (RIP)-1, which engages with the IκB kinase complex (17). Both TRAM and TRIF can also interact with TRAF-associated NF-κB activator (TANK) binding kinase-1, which phosphorylates and activates interferon regulatory factor 3 (18, 19).

Regarding tyrosine kinase activation by TLRs, LPS has been shown to activate the tyrosine kinases Src, Hck and Lyn, although their role in LPS signaling is somewhat uncertain (20, 21). Most recently we and others have shown that Bruton's tyrosine kinase (Btk), a member of the Tec family of protein tyrosine kinases, is a component of the TLR signaling pathway (22, 23). In particular, it has been shown that Btk interacts with the TIR domains of TLRs 4, 6, 8, and 9 and was also found to specifically associate with MyD88, Mal, and IL-1 receptor-associated kinase 1 (22). LPS induces tyrosine phosphorylation of Btk and activates its kinase activity, and monocytes from patients with X-linked agammaglobulinemia, which contain mutant Btk, are unresponsive to LPS (24).

A limited number of proteins have been shown to undergo tyrosine phosphorylation during TLR signaling. To date it has been reported that MyD88, TLR2, TLR3, and TLR4 become tyrosine phosphorylated during activation (25–27). In this study, we report that Mal is tyrosine phosphorylated during TLR2 and TLR4 signaling. We demonstrate that the tyrosine residues located at positions 86, 106, and 187 are likely to be phospho-accepting residues and that mutant forms of Mal in which Tyr-86 or Tyr-187 are substituted with phenylalanine act as dominant negative inhibitors of LPS signaling. Finally, we identify Btk as the responsible tyrosine kinase. Mal is therefore the first substrate for Btk in TLR signaling to be identified, its phosphorylation by Btk being an important mechanism in signaling by TLR2 and TLR4.

MATERIALS AND METHODS

Biological Reagents and Cell Culture—The HEK293 cell line was cultured in Dulbecco's modified Eagle's medium; the human monocytic THP 1 cell line was cultured in RPMI 1640 medium supplemented with

* This work was supported by Science Foundation Ireland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. Tel.: 353-1-6082439; Fax: 353-1-6772400; E-mail: laoneill@tcd.ie.

[2] The abbreviations used are: TLR, Toll like-receptor; TIR, Toll/interleukin-1 receptor; Btk, Bruton's tyrosine kinase; HEK, human embryonic kidney; LPS, lipopolysaccharide; MALP-2, macrophage-stimulating lipopeptide-2; Mal, MyD88 adapter-like; GST, glutathione *S*-transferase; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TRIF, TIR domain-containing adapter-inducing interferon; TRAM, TRIF-related adapter molecule.

10% (v/v) fetal calf serum. HEK293 cells stably expressing FLAG-tagged TLR4 were a generous gift from D. Golenbock (University of Massachusetts Medical School). Expression vectors encoding HA-Mal have been described elsewhere (28). The plasmid encoding the chimeric CD4-TLR4 was a kind gift from R. Medzhitov (Yale University School of Medicine). The NF-κB luciferase plasmid was a kind gift from R. Hofmeister (Universitaet Regensburg, Regensburg, Germany). The Btk-specific inhibitor LFM-A13 was obtained from Calbiochem. The anti-Mal antibody (Pearl-1) was obtained from Alexis Biochemicals. All other reagents were obtained from Sigma.

Immunoprecipitation and GST Pulldown Assays—HEK293 cells (1×10^6 cells) were harvested 24 h post-transfection. Lysis, immunoprecipitation, and GST pulldown assays were prepared as described elsewhere (22). For immunoprecipitation of phospho-Mal, cell lysates were incubated with anti-phosphotyrosine-agarose clone PT-66 overnight. After incubation, beads were washed, and the tyrosine phosphorylation status of Mal was analyzed by immunoblotting with an anti-Mal antibody.

Phosphatase Treatment—Immune complexes were incubated with 100 units of calf intestinal alkaline phosphatase with 30 μl of the phosphatase digestion buffer (50 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$). Samples were incubated at 37 °C for 3 h. For protein tyrosine phosphatase-1B treatment, the immune complexes were incubated with the phosphatase digestion buffer (50 mM imidazole, pH 7.5) at 37 °C for 30 min with 10 units of protein tyrosine phosphatase-1B.

In Vitro Kinase Assays—Immune complexes were incubated with 30 μl of kinase buffer (20 mM Hepes, pH 7.5, 2 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM NaCl, 100 μM Na_3VO_4, and 20 mM β-glycerolphosphate and protease inhibitors) containing 2 μCi of [γ-^{32}P]ATP and 0.6 μM of non-radioactive ATP. Samples were then incubated at 37 °C for 30 min. Gels were transferred onto polyvinylidene difluoride membranes and visualized by autoradiography.

Reporter Assays—HEK293 cells (2×10^4) were transfected with 5× NF-κB luciferase reporter gene plasmid and co-transfected with expression vectors using GeneJuice (Novagen). In all cases, 40 ng/well of phRL-TK reporter gene was co-transfected to normalize data for transfection efficiency. After 24 h, reporter gene activity was measured as described elsewhere (22). Data are expressed as the mean fold induction ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Two-dimensional Electrophoresis of Mal—Immunoprecipitations were performed as described above, and immune complexes were resuspended in 400 μl of sample solubilization solution (8 M urea, 50 mM dithiothreitol, 4% CHAPS, 0.2% carrier ampholytes, 0.0002% bromphenol blue). Immunoprecipitates were separated in the first dimension by isoelectric focusing performed using precast IPG strips (Immobiline™ DryStrip gels; Amersham Biosciences), pH 4–7. Focused proteins were separated in the second dimension by SDS-PAGE.

Modeling Studies—Modeling of the TIR domain of Mal was carried out as described previously (28).

RESULTS

Mal Is Tyrosine Phosphorylated—In our first analysis of Mal, we had observed in HEK293 cells transfected with a plasmid encoding Mal that in addition to the predominant 32-kDa form slower migrating forms of Mal could be detected following SDS-PAGE, suggesting that Mal may be covalently modified (3). As the isoelectric point of a protein is always altered once phosphorylation occurs, we examined the electrophoretic mobility of Mal by two-dimensional gel analysis. As we had observed previously, one-dimensional SDS-PAGE analysis revealed three distinct bands (Fig. 1*a*, *left panel*), whereas upon two-dimensional SDS-PAGE analysis two forms of Mal were detected by immunoblotting (*right panel*). We observed that the slower migrating form of Mal had a lower isoelectric point than the predominant 32-kDa form and is most likely an unresolved mixture of the two slower migrating forms detected on one-dimensional SDS-PAGE analysis. To further investigate the phosphorylation status of Mal, we treated immunoprecipitated Mal isolated from HEK293 cells overexpressing HA-Mal with calf intestinal phosphatase, which can remove phosphates from serine, threonine, and tyrosine residues (29). Treatment of Mal with calf intestinal alkaline phosphatase converted the slower migrating forms into the predominant 32-kDa form (Fig. 1*b*, *lane 2*), an effect that was blocked by the addition of phosphatase inhibitors (*lane 1*), indicating that the slower migrating forms represent phosphorylated Mal. To confirm that Mal could incorporate phosphate we carried out an *in vitro* kinase assay. As can be seen in Fig. 1*c*, *lane 1*, GST-Mal becomes phosphorylated by interacting with kinases in the lysate. GST alone is not phosphorylated (Fig. 1*c*, *lanes 2* and *4*), and Mal does not undergo autophosphorylation (Fig. 1*c*, *lane 3*).

To further define the particular phospho-accepting sites in Mal, we treated immunoprecipitated Mal isolated from HEK293 cells overexpressing HA-Mal with protein tyrosine phosphatase-1B, a phosphatase that specifically dephosphorylates phosphotyrosine residues. The slower migrating form of Mal was eliminated following phosphatase treatment, indicating that Mal is tyrosine phosphorylated (Fig. 1*d*, compare *lane 2* to *lane 1*). To further confirm that Mal was tyrosine phosphorylated, immunoprecipitated Mal isolated from HEK293 cells overexpressing HA-Mal was resolved by SDS-PAGE and immunoblotted with a phosphotyrosine-specific antibody (Fig. 1*e*, *lane 2*).

Identification of Tyr-86, Tyr-106, and Tyr-187 as Possible Phospho-acceptors—Human Mal contains six tyrosine residues, all of which are conserved in the mouse sequence, attesting to their possible importance in the functioning of Mal (Fig. 2*a*). One tyrosine, Tyr-86, is a conserved residue among all TIR domain-containing proteins and is located within Box 1, the signature sequence of the TIR domain. Tyr-106 is situated between Box 1 and Box 2, whereas the remaining four tyrosine residues at positions 159, 187, 195, and 196 are located distal to Box 2. To examine the role of these tyrosine residues in Mal phosphorylation, we mutated the six tyrosine residues conservatively to phenylalanine and analyzed the mobility pattern of the Mal tyrosine mutants. Interestingly, we found that the slowest migrating form of wild-type Mal was no longer evident in Mal-Y86F, Mal-Y106F, or Mal-Y187F (Fig. 2*b*, *lanes 2*, *3*, and *5*). The mutant proteins Y159F, Y195F, and Y196F retained the same electrophoretic mobility pattern as wild-type Mal (Fig. 2*b*, *lanes 4*, *6*, and *7*). These results suggested that the tyrosine residues located at positions 86, 106, and 187 were potential phosphorylation sites with all three tyrosine residues requiring phosphorylation for the altered mobility of Mal on SDS-PAGE. This was also apparent from the analysis of a triple mutant of these amino acids, Mal-Y86F,Y106F,Y187F, which gave the same expression profile as the single mutants (Fig. 2*b*, *lane 8*).

To assess the requirement for the tyrosine residues in Mal-mediated NF-κB activation, Mal mutant proteins were overexpressed in HEK293 cells in conjunction with an NF-κB-dependent luciferase reporter gene. In agreement with published results (3, 4), wild-type Mal strongly activated NF-κB (Fig. 2*c*). In contrast, the Mal mutant proteins Y86F, Y106F, and Y187F were less active, with Mal-Y86F and Mal-Y106F particularly impaired. Analysis of the ability of the triple mutant of Mal to activate NF-κB further confirmed that Tyr-86, Tyr-106, and Tyr-187 are critical residues for NF-κB activity induced by Mal. Mal-Y159F,

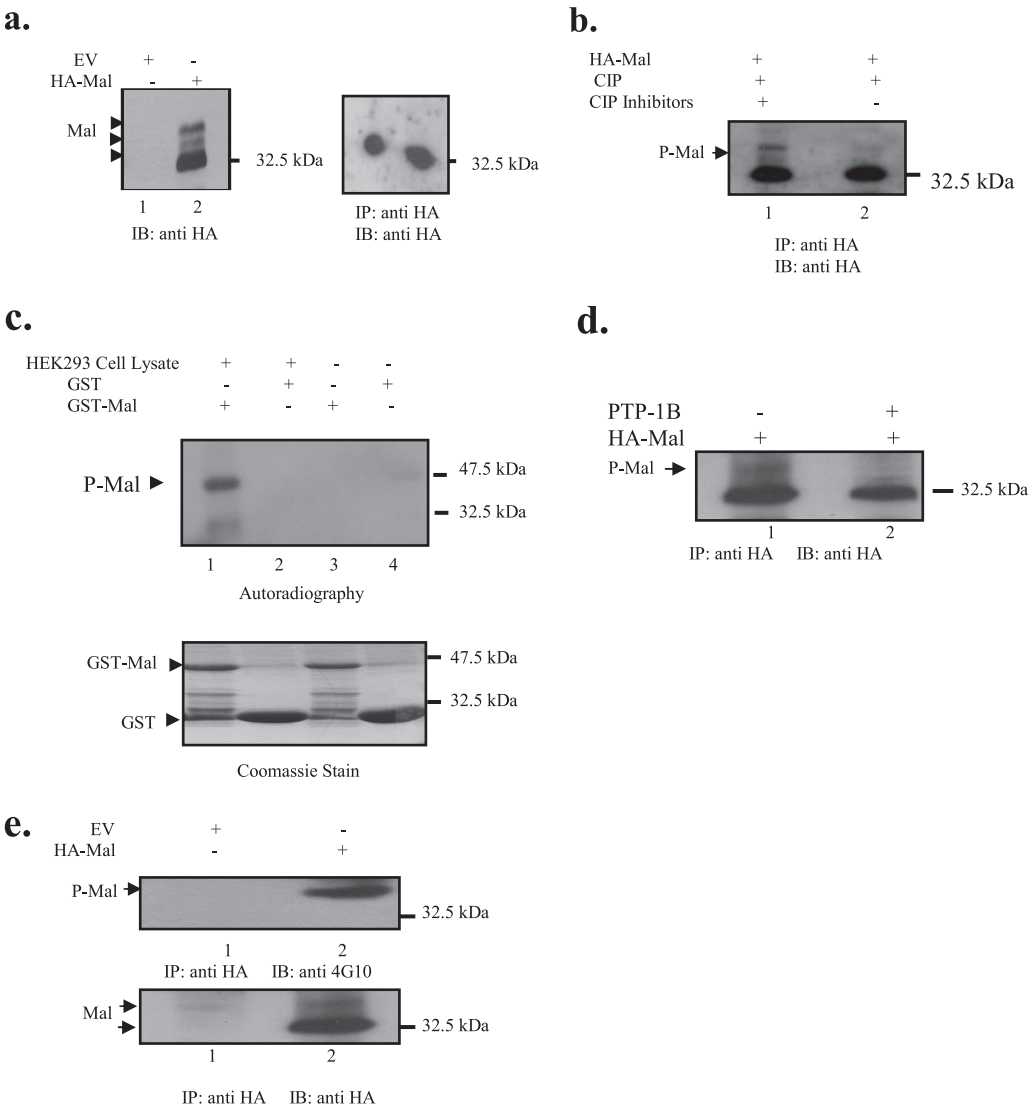

FIGURE 1. **Mal is a phosphoprotein.** *a*, *left panel*, HEK293 cells were transiently transfected with a plasmid encoding HA-Mal (*lane 2*) or empty vector (*EV*, *lane 1*). Cell lysates were prepared, and samples were immunoblotted with an anti-HA antibody. *Right panel*, two-dimensional gel analysis of the phosphorylation status of Mal. HEK293 cells were transiently transfected with a plasmid encoding HA-Mal. Cell lysates were prepared, and Mal was immunoprecipitated with an anti-HA antibody. The sample was analyzed by two-dimensional SDS-PAGE, followed by immunoblotting with an anti-HA antibody. The sample was electrofocused at a pH gradient of 4 (*left*) to 7 (*right*). *b*, phosphatase treatment results in disappearance of the slower migrating forms of Mal. HEK293 cells were transiently transfected with a plasmid encoding HA-Mal. Cell lysates were prepared, and Mal was immunoprecipitated with an anti-HA antibody. Immunoprecipitates were incubated at 37 °C for 3 h with 100 units of calf intestinal alkaline phosphatase in the presence (*lane 1*) or absence (*lane 2*) of calf intestinal alkaline phosphatase inhibitors. Samples were then analyzed by SDS-PAGE and immunoblotted with an anti-HA antibody. *c*, GST-Mal is phosphorylated but does not undergo autophosphorylation when subjected to an *in vitro* kinase assay. GST-Mal (*lane 1*) and GST (*lane 2*) were incubated with cell lysates from the HEK293 cell line or with lysis buffer alone (*lanes 3* and *4*) for 2 h at 4 °C. Samples were subjected to an *in vitro* kinase assay, separated by SDS-PAGE, and visualized by autoradiography (*upper panel*). Samples were also stained with Coomassie stain (*lower panel*). *d*, Mal is tyrosine phosphorylated. HA-Mal was immunoprecipitated from HEK293 cells as described in *panel b*. Immunoprecipitates were incubated with (*lane 2*) or without (*lane 1*) 10 units of protein tyrosine phosphatase-1B. Samples were analyzed by SDS-PAGE and immunoblotted with an anti-HA antibody. *e*, HEK293 cells (1×10^6) were mock-transfected (*lane 1*) or transiently transfected with a plasmid encoding HA-Mal (*lane 2*). HA-Mal was immunoprecipitated with an anti-HA antibody, and samples were immunoblotted with an antibody that recognizes phosphotyrosine residues (clone 4G10; Upstate). Results shown are representative of at least three separate experiments.

Mal-Y195F, or Mal-Y196F activated NF-κB to a similar level as wild-type Mal (Fig. 2*c*). These results imply that it is Mal phosphorylated on Tyr-86, Tyr-106, and Tyr-187 that is the active form of Mal when overexpressed.

We next analyzed a molecular model of the TIR domain of Mal, which we had previously constructed (28), to determine the location of Tyr-86, Tyr-106, and Tyr-187. We noted that both Tyr-86 and Tyr-106 appear to be surface exposed, whereas Tyr-187, just visible on the surface model, appears to be the least accessible (Fig. 2*d*). It has been reported that the backbone of the TIR domain is similar to that of CheY, a bacterial chemotaxis protein (30). Studies have shown that CheY undergoes phosphorylation and that this phosphorylation event triggers a conformational change, thereby regulating its association with other proteins (31). It is possible that phosphorylation of Mal on Tyr-86 or Tyr-106 is required to initiate a conformational change in the TIR domain of Mal, thereby exposing Tyr-187 for phosphorylation, leading to activation of downstream signals.

We next tested whether Mal-Y86F, Mal-Y106F, or Mal-Y187F could have a dominant negative effect in LPS signaling. As has been previously shown (3, 4), a mutant form of Mal in which proline 125 in Box 2 of the TIR domain is mutated to histidine (P125H) strongly inhibited activation of NF-κB mediated by TLR4 (Fig. 2*e*). Interestingly, activation of NF-κB mediated by LPS was also strongly inhibited by Mal-Y86F, suggesting that this tyrosine residue is critical for efficient TLR4 signaling

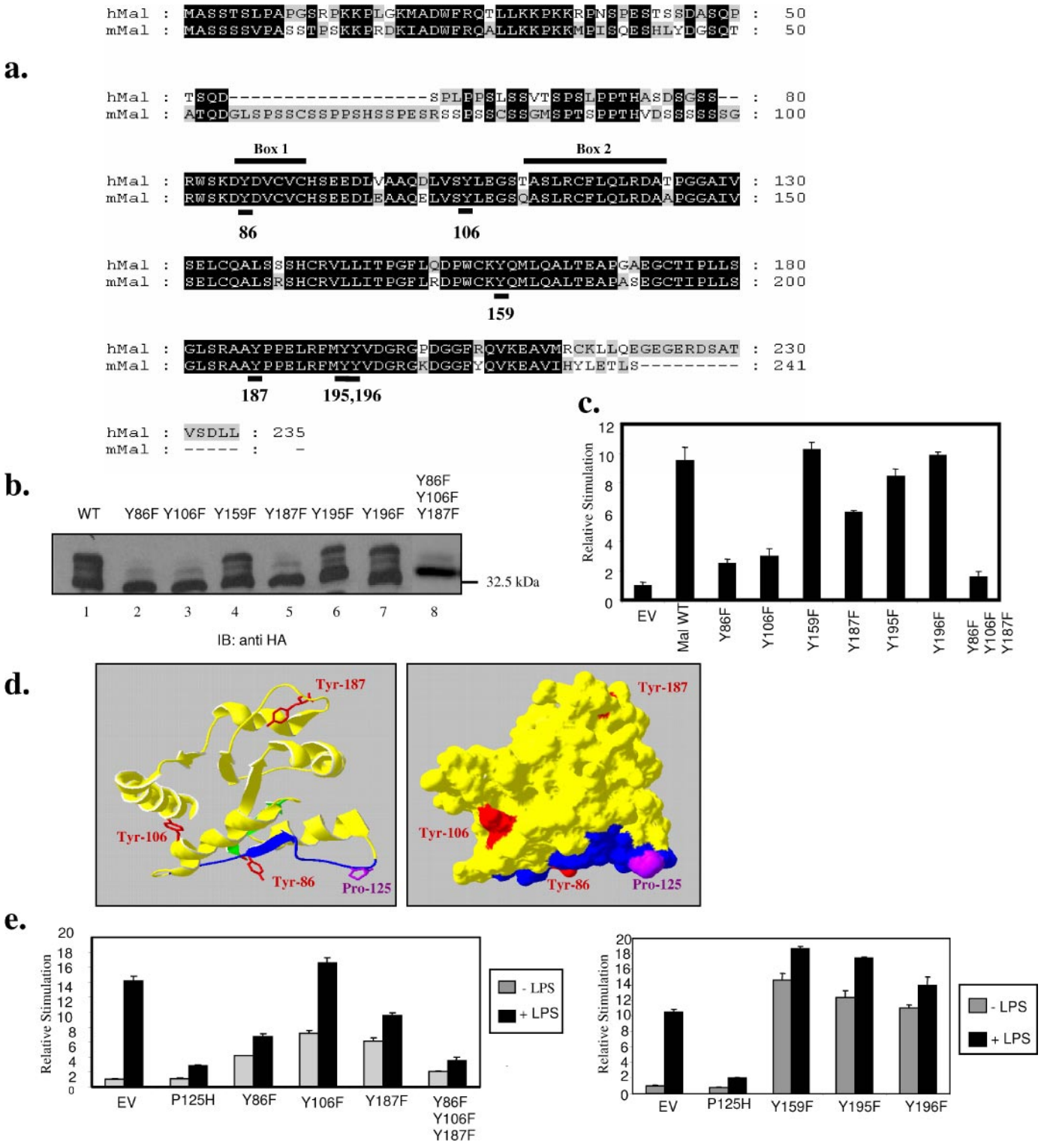

FIGURE 2. *a*, alignment of human Mal (*hMal*) and murine Mal (*mMal*). Two regions, *Box 1* and *Box 2*, which are conserved among all TIR domain-containing proteins, are indicated. The tyrosine residues conserved in both hMal and mMal are *underlined* and *numbered* according to their location in hMal. *b*, SDS-PAGE reveals that replacement of Tyr-86, -106, or -187 to Phe alters the phosphorylation profile of wild-type Mal. HEK293 cells were transiently transfected with plasmids encoding wild-type (*WT*) Mal (*lane 1*), Mal-Y86F (*lane 2*), Mal-Y106F (*lane 3*), Mal-Y159F (*lane 4*), Mal-Y187F (*lane 5*), Mal-Y195F (*lane 6*), Mal-Y196F (*lane 7*), and Mal-Y86F,Y106F,Y187F (*lane 8*) for 24 h. Lysates were resolved by SDS-PAGE and immunoblotted with an anti-HA antibody. *c*, Mal-Y86F, Mal-Y106F, and Mal-Y187F do not activate the NF-κB pathway as strongly as wild-type Mal. HEK293 cells were transiently transfected with a 5× NF-κB reporter gene plasmid and co-transfected with plasmids encoding wild-type Mal, Mal-Y86F, Mal-Y106F, Mal-Y159F, Mal-Y187F, Mal-Y195F, Mal-Y196F, and Mal-Y86F,Y106F,Y187F, for 24 h. Luciferase activity is expressed as fold induction relative to mock-transfected cells (*EV*). Mean relative stimulation of luciferase activity ± S.D. for a representative experiment from three separate experiments, each performed in triplicate, is shown. *d*, structural surface analysis of the TIR domain of Mal. Structural features representing the conserved boxes of the TIR domains are shown in *green* (*Box 1*) and *blue* (*Box 2*). The side chain of the semi-conserved proline residue in the BB-loop is colored *purple*. The phospho-accepting tyrosines Tyr-86, Tyr-106, and Tyr-187 are highlighted in *red*. *e*, Mal-Y86F and Mal-Y187F inhibit LPS signaling. HEK293 cells stably transfected with FLAG-TLR4 were transfected with a 5× NF-κB reporter gene plasmid and co-transfected with plasmids encoding Mal-P125H, Mal-Y86F, Mal-Y106F, Mal-159F, Mal-Y195F, Mal-Y196F and Mal-Y86F,Y106F,Y187F. Cells were then left untreated or incubated with LPS (100 ng ml^{-1}) for 6 h. Mean relative stimulation of luciferase activity ± S.D. for a representative experiment from three separate experiments, each performed in triplicate, is shown.

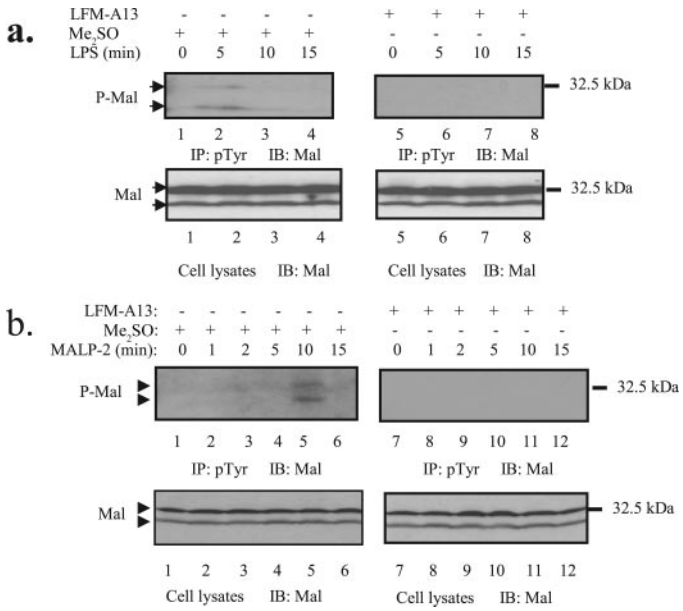

FIGURE 3. **Endogenous Mal is phosphorylated by Btk.** THP-1 cells (2×10^7) were pretreated with Me_2SO (*left side*) or LFM-A13 (*right side*) for 1 h prior to stimulation with either LPS (*a*) or MALP-2 (*b*). Cell lysates were prepared and tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine agarose, followed by immunoblotting with an anti-Mal antibody. Cell lysates were also immunoblotted with the anti-Mal antibody (*lower panels*).

(Fig. 2*e*). Mal-Y187F also blocked the LPS response albeit to a lesser degree. LPS could still stimulate NF-κB activation in cells expressing Mal-Y106F. Similar to Mal-P125H, the triple mutant Mal-Y86F,Y106F,Y187F severely inhibited NF-κB activation mediated by LPS. The Mal mutant proteins Y159F, Y195F, and Y196F were strongly active when overexpressed, and LPS treatment had no major influence on their effect. These results suggest that phosphorylation of Mal on Tyr-86 and Tyr-187 is required for Mal to signal NF-κB activation because these mutant forms inhibited the LPS response.

Phosphorylation of Mal by Btk—We next examined the tyrosine phosphorylation status of endogenous Mal in the human monocytic THP-1 cell line that expresses TLR2 and TLR4 and therefore responds to their respective stimuli, MALP-2 and LPS. Proteins phosphorylated on tyrosine residues were immunoprecipitated from THP-1 cells with an anti-phosphotyrosine antibody and immunoblotted with an anti-Mal antibody. Blotting for endogenous Mal revealed two distinct forms (Fig. 3, *a* and *b*, *left side*, *lower panels*). No tyrosine-phosphorylated Mal was detected in untreated cells, (Fig. 3, *a* and *b*, *upper panels*, *lanes 1*). Treatment of cells with LPS led to tyrosine phosphorylation of Mal, with two tyrosine-phosphorylated forms of endogenous Mal proteins being detected at 5 min-treatment time (Fig. 3*a*, *upper panel*, *lane 2*). Similar to LPS, the TLR2 ligand MALP-2 led to tyrosine phosphorylation of Mal, the effect being evident at 10-min post-stimulation (Fig. 3*b*, *upper panel*, *lane 5*), with two tyrosine-phosphorylated forms again evident. The effect of both LPS and MALP-2 was remarkably transient with no tyrosine phosphorylation detected at later times.

To date, Btk is the only tyrosine kinase known to interact with Mal (22). In this experiment, we therefore sought to determine whether Btk was involved in the tyrosine phosphorylation of Mal following stimulation of THP-1 cells with LPS or MALP-2. It was noted that pretreatment of THP-1 cells with LFM-A13, a specific Btk inhibitor, completely abolished LPS-induced tyrosine phosphorylation of Mal (Fig. 3*a*, *upper panel*, compare *lanes 2* and *6*). Similarly, MALP-2-induced tyrosine phosphorylation of Mal was totally abolished following pretreatment of THP-1 cells with LFM-A13 (Fig. 3*b*, *upper panel*, compare *lanes 5* and *11*).

To further confirm that Btk can phosphorylate Mal, *in vitro* kinase assays were performed. Incubation of recombinant Btk with recombinant Mal resulted in Mal phosphorylation, with two distinct phospho-forms appearing (Fig. 4*a*, *lane 2*). MyD88 was not phosphorylated by Btk under similar conditions (*lane 5*). Given that Tyr-86, Tyr-106, and Tyr-187 were possible phospho-acceptors, the effect of mutating these residues on Mal phosphorylation by Btk was next assessed. It was noted that phosphorylation of Mal by Btk was almost completely abolished when using recombinant Mal-Y86F,Y106F,Y187F as a substrate; furthermore, only one phospho-form was evident (Fig. 4*a*, *lane 3*). This experiment also revealed that Btk will undergo autophosphorylation as expected (*lanes 1* and *4*), but having Mal present limits this capacity as can be seen from decreased Btk autophosphorylation (compare *lanes 1* and *2*). Interestingly, Mal-Y86F,Y106F,Y187F altered the kinase activity of Btk to a greater extent than wild-type Mal (Fig. 4*a*, compare *lanes 2* and *3*). MyD88, however, does not alter the kinase activity of Btk (compare *lanes 4* and *5*).

We next performed *in vitro* kinase assays using endogenous Btk. We found that recombinant Mal is a substrate for endogenous Btk *in vitro*. Similar to previous studies, the activity of endogenous Btk was enhanced following LPS stimulation, with maximal *in vitro* activity being detected at 60 min as can be seen in Fig. 4*b*, *lane 4* (23). We observed that increased activity of Btk corresponded to an increase in the phosphorylation of recombinant Mal by Btk and that phosphorylation again resulted in the appearance of two phospho-forms. Taken together our results indicate that Mal undergoes phosphorylation on tyrosine residues by Btk during signaling, which is required for Mal to signal NF-κB activation.

DISCUSSION

Prompt activation of the TLR signaling pathway has proven to be vital in the recognition of invading pathogens and in eliciting the host innate immune response. Numerous studies have demonstrated that the efficient orchestration of the TLR signaling pathway is dependent on a series of phosphorylation events. The most receptor proximal protein kinases, which are recruited by TIR domain-containing adapters, are IL-1 receptor-associated kinases 4 and -1, RIP-1 and RIP-3, TANK binding kinase 1, and Btk (1). A limited number of substrates for these kinases have been reported to date. Here we have demonstrated that Mal undergoes tyrosine phosphorylation by Btk during signaling and that this phosphorylation event is required for Mal to signal NF-κB activation.

Two-dimensional SDS-PAGE analysis revealed that the slower migrating form of Mal displayed a lower isoelectric point than the predominant 32-kDa form. Phosphatase treatment established that the slower migrating form of Mal represented tyrosine-phosphorylated Mal. A role for tyrosine phosphorylation has previously been ascribed to the TLR2 and TLR4 signaling pathway (32, 33). In this study, MALP-2 and LPS rapidly triggered tyrosine phosphorylation of endogenous Mal in THP-1 cells.

We next assessed the relative functional importance of the six tyrosine residues in the TIR domain of Mal and noted that in Mal-Y86F, Mal-Y106F, and Mal-Y187F the slowest migrating form of Mal was abolished. Our findings also determined that Tyr-86, Tyr-106, and Tyr-187 were the most critical residues for maximal NF-κB activation induced by Mal. Furthermore, Mal-Y86F and Mal-Y187F acted as dominant negative inhibitors of NF-κB activation induced by LPS, whereas the other mutants of Mal did not. These results suggest that following

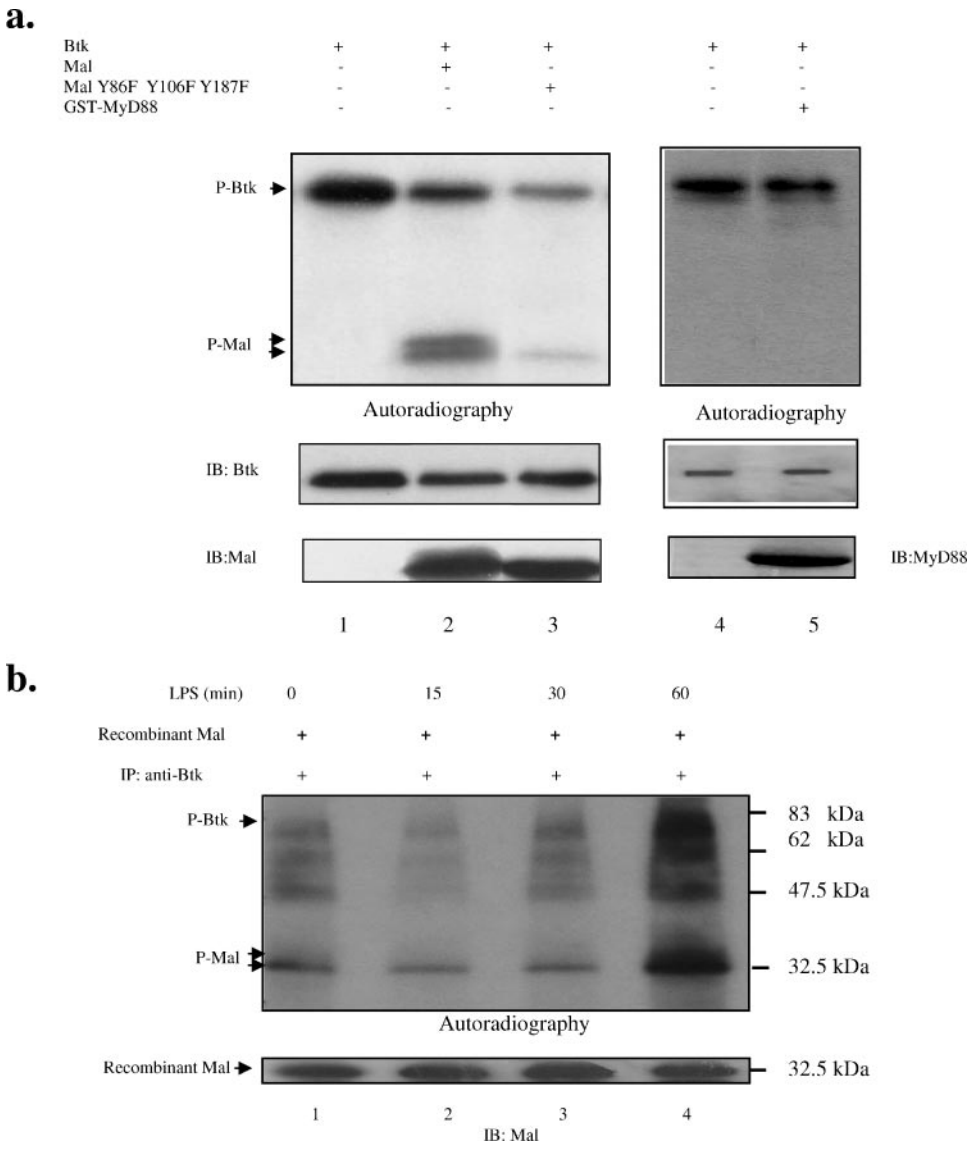

FIGURE 4. **Mal is a substrate for Btk.** *a*, recombinant Btk was incubated alone (*lanes 1* and *4*) or with recombinant wild-type Mal (*lane 2*), recombinant Mal-Y86F,Y106F,Y187F (*lane 3*), or recombinant GST-MyD88 (*lane 5*). Samples were subjected to an *in vitro* kinase assay, analyzed on SDS-PAGE, and visualized by autoradiography. Samples were also immunoblotted with either an anti-Btk antibody (*middle panel*), an anti-Mal antibody (*left lower panel*) or with an anti-MyD88 antibody (*right lower panel*). *b*, THP1 cells were treated with LPS (1 μg/ml) as indicated, cell lysates were prepared, and immunoprecipitations were performed using an anti-Btk antibody. Recombinant Mal was added to each sample. *In vitro* kinase assays were performed, and samples were analyzed by SDS-PAGE and visualized by autoradiography (*upper panel*). Samples were also immunoblotted with an anti-Mal antibody (*lower panel*).

LPS stimulation Tyr-86 and Tyr-187 are likely to undergo phosphorylation and this process is required for NF-κB activation mediated by Mal. It is likely that both require phosphorylation because mutating either resulted in an inactive dominant negative form.

Although Tyr-106 failed to activate NF-κB to the same extent as wild-type Mal and had a different phosphorylation profile to wild-type Mal, it had no effect on LPS signaling. All three tyrosines located at positions 86, 106, and 187 may need to undergo phosphorylation for Mal to exhibit retarded mobility. Given that during TLR2 and TLR4 signaling two tyrosine-phosphorylated forms of Mal could be detected, it is likely that endogenous Mal is also multiply tyrosine phosphorylated. The role of Tyr-106 in Mal signaling is therefore unclear but may be required for another Mal-mediated signal. We are currently examining this possibility.

We have previously shown that Btk is activated by TLR4 and is required for LPS responses mediating NF-κB activation (22). A particular response to LPS that requires Mal is phosphorylation of the p65 subunit of NF-κB at serine 536 (36). This response is abolished in cells from X-linked immunodeficiency mice, which contain inactive Btk (34), and is also inhibited by the Btk inhibitor LFM-A13 (22). Our findings here have clearly demonstrated that phosphorylation of Mal was enhanced upon increased activation of Btk following LPS stimulation. Mutational analysis of Mal indicates that Btk phosphorylates Mal on Tyr-86, Tyr-106, and Tyr-187. This observation therefore identifies Mal as a substrate for Btk on the TLR4 pathway, with phosphorylation of Mal on Tyr-86 and Tyr-187 by Btk being required for NF-κB activation mediated by LPS. Interestingly, we observed that Mal inhibited Btk autophosphorylation, suggesting that Mal may be required to negatively regulate the kinase activity of Btk. We attempted to confirm that Btk is a key tyrosine kinase for Mal phosphorylation by examining the phosphorylation status of Mal in monocytes and splenocytes isolated from X-linked immunodeficiency mice. The level of Mal, however, in cells from both wild-type and X-linked immunodeficiency mice was too low for detection in the endogenous phosphotyrosine immunoprecipitation assay. However, the inhibitory effect of LFM-A13 and the *in vitro* evidence strongly suggest that the tyrosine kinase responsible for tyrosine phosphorylation of Mal is Btk.

The tyrosine at position 86 is conserved among other TIR domain-containing proteins. It is therefore possible that Btk will phosphorylate the homologous tyrosine in other adapter proteins, although we found that MyD88 was not a substrate for Btk. We are currently exploring other TIR domain-containing proteins, notably the other adapters.

Recently, other proteins in the TLR signaling family have been shown to undergo phosphorylation. It has been reported that following stimulation with their specific ligands TLR2, TLR3, and TLR4 all undergo tyrosine phosphorylation (25–27). In addition, LPS was found to induce tyrosine phosphorylation of MyD88, and it has been stated that upon overexpression, the adapters TRIF and TRAM are phosphorylated (12, 19, 35). Although it has been established that members of the TLR superfamily undergo phosphorylation, the participating kinases have yet to be identified, and to date their identity has only been speculated upon. Therefore, this report is the first to identify a kinase that directly phosphorylates a TIR domain-containing protein.

Our study has not addressed the immediate downstream consequences of tyrosine phosphorylation of Mal. Indeed, similar to CheY, which is structurally similar to the TIR domain, a conformational change for signaling may occur following phosphorylation of Mal and specific proteins might be recruited such as suppressor of cytokine signaling-1 (36). We are currently examining these possibilities. Our results, however, clearly show that Mal is a phosphoprotein that becomes tyrosine phosphorylated by Btk upon activation of the TLR2 and TLR4 signal transduction pathways. This phosphorylation is required for Mal to signal, establishing an important mechanistic step in TLR2 and TLR4 signaling.

Acknowledgment—We thank M. Ejdebäck for constructing the molecular model of the TIR domain of Mal.

REFERENCES

1. Dunne, A., and O'Neill, L. A. (2005) *FEBS Lett.* **579,** 3330–3335
2. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) *Nature* **413,** 732–738
3. Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O'Neill, L. A. (2001) *Nature* **413,** 78–83
4. Horng, T., Barton, G. M., and Medzhitov, R. (2001) *Nat. Immunol.* **2,** 835–841
5. Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002) *Nature* **420,** 329–333
6. Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K., and Akira, S. (2002) *Nature* **420,** 324–329
7. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 115–122
8. Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Morr, M., Muhlradt, P. F., and Akira, S. (2000) *J. Immunol.* **164,** 554–557
9. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) *J. Immunol.* **169,** 6668–6672
10. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) *Nat. Immunol.* **4,** 161–167
11. Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M., and Golenbock, D. T. (2003) *J. Exp. Med.* **198,** 1043–1055
12. Bin, L. H., Xu, L. G., and Shu, H. B. (2003) *J. Biol. Chem.* **278,** 24526–24532
13. Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M., and Seya, T. (2003) *J. Biol. Chem.* **278,** 49751–49762
14. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W. C. (2002) *Nature* **416,** 750–756
15. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) *Nature* **398,** 252–256
16. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) *Nature* **412,** 346–351
17. Meylan, E., Burns, K., Hofmann, K., Blancheteau, V., Martinon, F., Kelliher, M., and Tschopp, J. (2004) *Nat. Immunol.* **5,** 503–507
18. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) *Nat. Immunol.* **4,** 491–496
19. Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K., and Akira, S. (2003) *J. Immunol.* **171,** 4304–4310
20. Napolitani, G., Bortoletto, N., Racioppi, L., Lanzavecchia, A., and D'Oro, U. (2003) *Eur. J. Immunol.* **33,** 2832–2841
21. Beaty, C. D., Franklin, T. L., Uehara, Y., and Wilson, C. B. (1994) *Eur. J. Immunol.* **24,** 1278–1284
22. Jefferies, C. A., Doyle, S., Brunner, C., Dunne, A., Brint, E., Wietek, C., Walch, E., Wirth, T., and O'Neill, L. A. (2003) *J. Biol. Chem.* **278,** 26258–26264
23. Mukhopadhyay, S., Mohanty, M., Mangla, A., George, A., Bal, V., Rath, S., and Ravindran, B. (2002) *J. Immunol.* **168,** 2914–2921
24. Horwood, N. J., Mahon, T., McDaid, J. P., Campbell, J., Mano, H., Brennan, F. M., Webster, D., and Foxwell, B. M. (2003) *J. Exp. Med.* **197,** 1603–1611
25. Chen, L. Y., Zuraw, B. L., Zhao, M., Liu, F. T., Huang, S., and Pan, Z. K. (2003) *Am. J. Physiol.* **284,** L607-L613
26. Sarkar, S. N., Smith, H. L., Rowe, T. M., and Sen, G. C. (2003) *J. Biol. Chem.* **278,** 4393–4396
27. Arbibe, L., Mira, J. P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P. J., Ulevitch, R. J., and Knaus, U. G. (2000) *Nat. Immunol.* **1,** 533–540
28. Dunne, A., Ejdeback, M., Ludidi, P. L., O'Neill, L. A., and Gay, N. J. (2003) *J. Biol. Chem.* **278,** 41443–41451
29. Ben-Yehoyada, M., Ben-Dor, I., and Shaul, Y. (2003) *J. Biol. Chem.* **278,** 34475–34482
30. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 588–593
31. Drake, S. K., Bourret, R. B., Luck, L. A., Simon, M. I., and Falke, J. J. (1993) *J. Biol. Chem.* **268,** 13081–13088
32. Hazeki, K., Masuda, N., Funami, K., Sukenobu, N., Matsumoto, M., Akira, S., Takeda, K., Seya, T., and Hazeki, O. (2003) *Eur. J. Immunol.* **33,** 740–747
33. Geng, Y., Gulbins, E., Altman, A., and Lotz, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8602–8606
34. Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., and Jenkins, N. A., and Witte, D. N. (1993) *Science* **261,** 358–361
35. Ojaniemi, M., Glumoff, V., Harju, K., Liljeroos, M., Vuori, K., and Hallman, M. (2003) *Eur. J. Immunol.* **33,** 597–605
36. Mansell, A., Smith, R., Doyle, S. L., Gray, P., Fenner, J. E., Crack, P. J., Nicholson, S. E., Hilton, D. J., O'Neill, L. A., and Hertzog, P. J. (2006) *Nat. Immunol.* **7,** 148–155