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

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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 monocytes 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) 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 cystolic 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 receptors, 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...
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10% (v/v) fetal calf serum. HEK293 cells stably expressing FLAG-tagged TLR4 were a generous gift from D. Golembek (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 (Universitätsmedizin, 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.

**RESULTS**

**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. 2a). 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. 2b, lanes 2, 3, and 5). The mutant proteins Y159F, Y195F, and Y196F retained the same electrophoretic mobility pattern as wild-type Mal (Fig. 2b, lanes 4, 6, and 7). These results suggested that the tyrosine residues located at positions 86, 106, and 187 were potential phosphoacceptors that mediate the mobility pattern of Mal tyrosine mutants. Mal-Y159F, Mal-Y106F, and Mal-Y187F, which gave the same expression profile as the single mutants (Fig. 2b, lane 8).

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

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. 2c). 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,
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 electrophoresed 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. 2c). 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. 2d). 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. 2e). 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.

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-Y159F, 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.
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. 3a, 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. 3b, 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 phosphoforms appearing (Fig. 4a, 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. 4a, lane 2). 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. 4a, 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. 4b, 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 effect of mutating these residues on Mal phosphorylation by Btk was almost completely abolished. Our findings also determined that Tyr-86, Tyr-106, and Tyr-187 were the most critical residues for maximal NF-κB activation.

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FIGURE 3. Endogenous Mal is phosphorylated by Btk. THP-1 cells (2 × 10⁶) were pretreated with Me2SO (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).
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 (35). 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.

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