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Myeloid Differentiation Factor-2 Interacts with Lyn Kinase and Is Tyrosine Phosphorylated Following Lipopolysaccharide-Induced Activation of the TLR4 Signaling Pathway

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Stimulation with LPS induces tyrosine phosphorylation of numerous proteins involved in the TLR signaling pathway. In this study, we demonstrated that myeloid differentiation factor-2 (MD-2) is also tyrosine phosphorylated following LPS stimulation. LPS-induced tyrosine phosphorylation of MD-2 is specific; it is blocked by the tyrosine kinase inhibitor, herbimycin A, as well as by an inhibitor of endocytosis, cytochalasin D, suggesting that MD-2 phosphorylation occurs during trafficking of MD-2 and not on the cell surface. Furthermore, we identified two possible phospho-accepting tyrosine residues at positions 22 and 131. Mutant proteins in which these tyrosines were changed to phenylalanine had reduced phosphorylation and significantly diminished ability to activate NF-κB in response to LPS. In addition, MD-2 coprecipitated and colocalized with Lyn kinase, most likely in the endoplasmic reticulum. A Lyn-binding peptide inhibitor abolished MD-2 tyrosine phosphorylation, suggesting that Lyn is a likely candidate to be the kinase required for MD-2 tyrosine phosphorylation. Our study demonstrated that tyrosine phosphorylation of MD-2 is important for signaling following exposure to LPS and underscores the importance of this event in mediating an efficient and prompt immune response. The Journal of Immunology, 2011, 187: 4331–4337.

We recently identified and described a novel alternatively spliced isoform of human MD-2, which lacks the region encoded by exon 2 of the MD-2 gene and showed that it is upregulated by IFN-γ, IL-6, and TLR4 stimulation; is a negative regulator of LPS-mediated TLR4 signaling; and competitively inhibits binding of full-length MD-2 to TLR4 (8).

Upon ligand binding, the TLR signaling pathway initiates a cascade of serine, threonine, and tyrosine phosphorylation events. Interestingly, several members of the TLR family are also tyrosine phosphorylated, including TLR2 (9), TLR3 (10–12), and TLR4 (13, 14). The identity of the kinases involved have yet to be elucidated; however, in the case of TLR4, the Src kinase Lyn was implicated in this posttranslational modification (13). In addition to TLRs, the TLR adapter proteins, MyD88 (15), MyD88-adapter like (16), TRIF (17), and TRAM (18) were shown to be phosphorylated.

In this study, we identified that MD-2 is also tyrosine phosphorylated upon LPS binding. This phosphorylation event is inhibited by the tyrosine kinase inhibitor herbimycin A. Furthermore, an endocytosis inhibitor, cytochalasin D, could block the tyrosine phosphorylation of MD-2 in cells stimulated with LPS. We identified two residues, located at positions 22 and 131, as possible phospho-accepting tyrosines. Mutant proteins in which these tyrosines were altered to phenylalanine have less phosphorylation and a significantly reduced ability to activate LPS-induced NF-κB and IL-8. In addition, we determined that Lyn interacts with MD-2 and that a Lyn-binding peptide inhibitor specifically abolishes MD-2 tyrosine phosphorylation, indicating that Lyn is the likely kinase required for MD-2 tyrosine phosphorylation. Our study is the first, to our knowledge, to identify MD-2 as a phosphoprotein and demonstrates the importance of this posttranslational event as a mechanism required for MD-2–TLR4–LPS signaling.

Materials and Methods

Cell culture and biological reagents

The HEK293 cell line was cultured in DMEM, supplemented with 10% heat-inactivated FBS and 2 mM glutamine. LPS (TLR4-grade) was from...
Alexis. Protein tyrosine kinase inhibitor herbimycin A was purchased from Sigma-Aldrich. Lyn peptide inhibitor was purchased from Tocris Cookson. 4G10 anti-phosphotyrosine Ab was purchased from Upstate. Anti-Flag agarose affinity gel and anti-Flag Ab were from Sigma Aldrich. Anti-calnexin, anti-Myc and anti-Lyn were from Santa Cruz Biotechnology. Anti-hemagglutinin (HA), Alexa Fluor 594 anti-rabbit, Alexa Fluor 488 anti-mouse, and Alexa Fluor 647 anti-goat IgG were from Invitrogen.

Immunoprecipitation

HEK293 cells were seeded into 100-mm dishes (1.5 × 10⁶) 24 h prior to transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen). For coimmunoprecipitations, 4 µg each construct was transfected. The total amount of DNA in each sample was kept constant by using empty-vector cDNA. Cells were harvested 24 h after transfection in 600 µl lysis buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40 containing protease inhibitor mixture, and 1 mM sodium orthovanadate). For immunoprecipitation, anti-Flag M1 agarose affinity gel or anti-Myc Ab with TrueBlot anti-mouse IgG beads (eBioscience) was incubated with the cell lysates overnight at 4°C. The immune complexes were then washed, and the associated proteins were eluted from the beads by boiling in 35 µl sample buffer and fractionated by SDS-PAGE.

Fluorescent microscopy

For immunoblotting, primary Abs were incubated overnight and detected using HRP-conjugated secondary Abs, followed by ECL (Amersham Biosciences).

Reporter gene assays

HEK293 cells were transiently transfected with plasmids expressing Flag–MD-2. After 24 h, cells were fixed with 4% paraformaldehyde (Electron Microscopy Science) in PBS, permeabilized using 0.2% Triton X-100 (Sigma), and blocked with DakoCytomation protein block (Dako). Cells were then incubated with primary Abs overnight at 4°C and with secondary Abs for 1 h at room temperature. Cells were mounted on slides using Prolong with DAPI (Invitrogen). Images were acquired in independent channels with a Zeiss ApoTome-equipped fluorescence microscope.

Immunoblotting

For immunoblotting, primary Abs were incubated overnight and detected using HRP-conjugated secondary Abs, followed by ECL (Amersham Biosciences).

ELISA

HEK293 cells, overexpressing TLR4, mutant MD-2 proteins, and CD14, were pretreated or not with the Lyn peptide inhibitor for 2 h prior to LPS stimulation, and supernatants were collected. Levels of IL-8 were determined by ELISA (BD Biosciences).

Results

MD-2 is tyrosine phosphorylated upon stimulation with LPS

Tyrosine phosphorylation of numerous proteins is induced upon stimulation with LPS; indeed, the LPS receptor TLR4 is tyrosine phosphorylated. Given the important role of MD-2 following LPS stimulation, we investigated whether it is posttranslationally modified as well. HEK293 cells were transiently transfected with plasmids expressing Myc-TLR4, Flag–MD-2, and CD14 or were mock transfected. Twenty-four hours later, cells were left untreated or were stimulated with LPS for various lengths of time. Proteins were immunoprecipitated from cell extracts with an anti-Flag Ab and analyzed by immunoblotting with an Ab that detects proteins phosphorylated on phosphotyrosine residues. Upon stimulation with LPS, MD-2 is tyrosine phosphorylated (Fig. 1A). The presence of 5 mM phosphotyrosine, a competitive inhibitor, completely abrogated the immunoreactivity detected by the anti-phosphotyrosine

Ab; in contrast, phosphosorine or phosphothreonine had no effect on MD-2 tyrosine phosphorylation (data not shown), which confirmed the specificity of this result. In addition, MD-2 tyrosine phosphorylation was not observed after stimulation with IL-1β, TNF-α, or Rhodobacter sphaeroides diphosphoryl lipid A, a biologically inactive analog of lipid A (data not shown). To further confirm that MD-2 is posttranslationally modified, we pretreated HEK293 cells, overexpressing TLR4 and MD-2, with the tyrosine kinase inhibitor herbimycin A for 2 h prior to LPS stimulation; herbimycin A significantly inhibited LPS-induced MD-2 tyrosine phosphorylation in a dose-dependent manner (Fig. 1B, compare lanes 3 and 4 with lane 2). We next hypothesized that MD-2 phosphorylation must occur during trafficking and not on the cell surface; therefore, we investigated the role of receptor or ligand internalization and endocytosis on the phosphorylation status of MD-2. Because cytochalasins effectively block LPS internalization and signaling for cytokine release (19), we pretreated HEK293 cells, overexpressing TLR4 and MD-2, with cytochalasin D for 1 h prior to LPS stimulation. We observed that LPS-induced MD-2 tyrosine phosphorylation was significantly inhibited following pretreatment with cytochalasin D (Fig. 1B, lane 5). This suggested that MD-2 tyrosine phosphorylation most likely occurs intracellularly during trafficking and not on the cell surface.

Identification of Tyr23 and Tyr131 as possible phospho-acceptors

Human MD-2 contains nine tyrosine residues (Fig. 2A). We mutated all of these residues conservatively to phenylalanine and tested their ability to respond to LPS. HEK293 cells stably transfected with an NF-κB reporter gene and TLR4 were transiently transfected with plasmids encoding wild-type MD-2, Y22F, Y34F, Y36F, Y42F, Y65F, Y75F, Y79F, Y102F, or Y131F. Twenty-four hours later, cells were stimulated with LPS. As can be seen in Fig. 2B, upon LPS stimulation, the mutant proteins Y22F and Y131F were significantly less potent in their ability to activate NF-κB compared with wild-type MD-2. Analysis of a double-mutant protein, Y22F+Y131F, confirmed that
these residues are important for MD-2 to signal NF-κB activation in response to LPS (Fig. 2C). We also determined that the mutant proteins lacking tyrosine residues located at positions 22 and 131 had a diminished ability to activate IL-8 (Fig. 2D). We further characterized these mutant MD-2 proteins by analyzing their phosphorylation status after LPS stimulation. Supporting our prediction that the sites we mutagenized were phosphorylation accepting residues, we next determined the location of these residues with respect to the published crystal structure of MD-2. As shown in Fig. 3A and 3C, the hydroxyl groups of both residues seem to be surface exposed, thereby allowing phosphorylation of these tyrosine residues to occur. Although Tyr$^{131}$ is located at the hydrophobic pocket of MD-2, neither Tyr$^{22}$ nor Tyr$^{131}$ is involved in the main dimerization interface of the TLR4–MD-2–LPS complex (Fig. 3B, 3D).

**MD-2 interacts with Lyn**

Prior studies showed that the Src kinase, Lyn, is recruited to TLR4 (13), as well as to CD14 (20). Furthermore, it was suggested that Lyn may be involved in TLR4 tyrosine phosphorylation. Similar to previous results, we found that TLR4 immunoprecipitated with Lyn upon LPS stimulation (Fig. 4A). Therefore, we investigated whether Lyn was also involved in the phosphorylation of MD-2, given that MD-2 tyrosine phosphorylation was abolished following pretreatment with herbimycin A (Fig. 1B), a potent Src kinase inhibitor, which has been shown to inhibit Lyn activity (21). HEK293 cells were transiently transfected with Myc-TLR4, Flag–MD-2, and CD14 constructs. Lyn was immunoprecipitated from HEK293 cells with plasmids expressing HA–MD-2 and Flag-Lyn inhibitor, which has been shown to inhibit Lyn activity (21).

HEK293 cells were transiently transfected with Myc-TLR4, Flag–MD-2, and CD14 constructs. Lyn was immunoprecipitated from HEK293 cells with plasmids expressing HA–MD-2 and Flag-Lyn inhibitor, which has been shown to inhibit Lyn activity (21).
Colocalization of endogenous Lyn and MD-2 in endoplasmic reticulum

Because we found that Lyn kinase and MD-2 could directly interact with each other in HEK293 cells (Fig. 4B, 4C), we also investigated whether endogenous Lyn and transfected MD-2 would colocalize, as determined by confocal microscopy. HEK293 cells were transiently transfected with plasmids expressing Flag–MD-2 and Myc-TLR4. After 24 h, cells were fixed and stained with anti-Lyn (red) and anti-Flag (green) Abs and nuclear stain DAPI (blue). Yellow staining in the merged image indicates colocalization between Lyn kinase and MD-2 (Fig. 5A). Further investigations revealed that the colocalization of endogenous Lyn with MD-2 was most likely in the endoplasmic reticulum (ER) (Fig. 5B). Finally, the addition of LPS to the cells did not alter the colocalization of Lyn kinase with MD-2 (Fig. 5C).

Lyn-specific peptide inhibitor blocks MD-2 tyrosine phosphorylation

Given that MD-2 tyrosine phosphorylation was abolished following pretreatment with herbimycin A and that MD-2 and Lyn were found to be in a complex together, we next wanted to further confirm that Lyn was required for MD-2 tyrosine phosphorylation. Therefore, we used a Lyn-specific peptide inhibitor (22) to examine the effect of inhibiting Lyn activation on MD-2 phosphorylation. HEK293 cells, overexpressing TLR4, Flag–MD-2, and CD14, were pretreated with the Lyn peptide inhibitor for 2 h prior to LPS stimulation. As can be seen in Fig. 6A, the Lyn peptide inhibitor significantly abolished LPS-induced MD-2 tyrosine phosphorylation in a dose-dependent manner (Fig. 6A, compare lanes 4–6 with lane 3). Furthermore, following pretreatment with the Lyn peptide inhibitor, LPS-induced activation of IL-8 and NF-κB was also abolished in HEK293 cells overexpressing TLR4, CD14, and MD-2 (Fig. 6B). Additionally, we measured the protein levels of IL-8 (Fig. 6C). HEK293 cells, overexpressing TLR4, CD14, and wild-type or mutant MD-2 proteins, were pretreated or not with the Lyn peptide inhibitor for 2 h prior to LPS stimulation. IL-8 production was significantly diminished in cells transfected with wild-type MD-2 and the nonaffected mutants Y75F and Y79F, whereas there was no significant difference with or without Lyn peptide inhibitor in the phosphorylation mutants Y22F and Y131F, as well as in Y22F+Y131F double-mutant–transfected cells, thus confirming the importance of MD-2 tyrosine phosphorylation by Lyn kinase in mediating the signaling of the LPS–MD-2–TLR4 complex.
Discussion

It is apparent that LPS requires both TLR4 and MD-2 to initiate signal transduction and innate immune responses, in addition to LPS binding protein (2) and CD14 (3). Furthermore, crystal structural data for the TLR4–MD-2 complex bound to hexa-acyl lipid A showed that MD-2 directly binds the lipid A portion of LPS with high avidity, because its hydrophobic pocket can accommodate up to five lipid A acyl chains (7). In this study, we further clarified the role of MD-2 in TLR4 signaling and demonstrated that MD-2 undergoes tyrosine phosphorylation upon LPS stimulation and that this phosphorylation event is required for LPS-induced NF-κB activation.

Excessive inflammation is the hallmark of a number of infectious pathologies, such as sepsis, acute respiratory distress syndrome, and multiple organ failure (23); therefore, tight regulation of the TLR4 signaling pathway at multiple levels is imperative to prevent an overactivated immune response. Phosphorylation is a highly conserved mechanism that can be used to regulate protein function. Indeed, several studies illustrated that the TLR signaling pathway is dependent on a series of phosphorylation events. In this study, we discovered that similar to TLR2, TLR3, and TLR4, MD-2 undergoes tyrosine phosphorylation in response to LPS. Furthermore, our results suggested that phosphorylation of MD-2 on specific tyrosines is required for NF-κB activation and may be a regulatory step used to curtail an overexuberant host immune response.

The LPS–TLR4–MD-2 complex is rapidly endocytosed upon initiation of signaling and can be located in endosomes, Golgi apparatus, and lysosomes (24, 25). Cytochalasin D, a potent actin polymerization inhibitor that prevents LPS endocytosis (19), inhibited LPS-induced tyrosine phosphorylation of MD-2, suggesting that LPS-induced MD-2 phosphorylation occurs in intracellular compartments following internalization and trafficking of the LPS–TLR4–MD-2 complex and not on the cell surface. Furthermore, we confirmed that this MD-2 tyrosine phosphorylation was specific to LPS stimulation, indicating that these residues were critical for maximal NF-κB activation and might be phospho-accepting.

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FIGURE 5. Colocalization of endogenous Lyn with MD-2. HEK293 cells were transiently transfected with plasmids expressing Flag–MD-2. After 24 h, cells were immunostained with anti-Lyn (red) and anti-Flag (green) Abs and nuclear stain DAPI (blue). Some samples were also stained with anti-calnexin (far-red).

A. Colocalization of endogenous Lyn with MD-2 (yellow-merged image).

B. Lyn and MD-2 colocalize with ER (white-merged image).

C. Lyn and MD-2 colocalize with ER (white-merged image) in the presence of LPS. Original magnification ×63.

FIGURE 6. MD-2 tyrosine phosphorylation is inhibited by Lyn peptide inhibitor. A. HEK293 cells were transiently transfected with TLR4 and Flag–MD-2 (lanes 1–6). Twenty-four hours later, cells were pretreated for 2 h with Lyn peptide inhibitor at 10, 20, or 30 μM, prior to stimulation with LPS for 10 min (lanes 3–6). Flag-tagged proteins were immunoprecipitated with an anti-Flag Ab in cell lysates, analyzed by SDS-PAGE, and immunoblotted with an anti-phosphotyrosine Ab (top panel) or anti-Flag Ab (bottom panel). B. HEK293 cells stably transfected with an NF-κB or IL-8 reporter gene and TLR4 were transiently transfected with wild-type MD-2. Twenty-four hours later, cells were pretreated for 2 h with Lyn peptide inhibitor (mM) at indicated doses. Cells were left untreated or stimulated with LPS for 6 h, and luciferase activity was measured in cell lysates and expressed as fold induction relative to untreated cells.

C. HEK293 cells were transiently transfected with TLR4, wild-type MD-2, Y75F, Y79F, Y22F, Y131F, or Y22F+Y131F constructs. Twenty-four hours later, cells were pretreated or not for 2 h with Lyn peptide inhibitor (20 μM) and stimulated with LPS for 6 h. IL-8 levels in the supernatants were measured by ELISA. *p < 0.05.
residues. In addition, by analyzing the published crystal structure of MD-2, we determined that the hydroxyl groups of both MD-2 tyrosine residues, located at positions 22 and 131, are surface exposed, thereby permitting their phosphorylation. Activation of Lyn kinase activity is a very early event in LPS signaling in monocytes (26). Lyn kinase is also a candidate kinase for MD-2 phosphorylation. Lyn kinase is recruited to TLR4, as well as to CD14 (20), upon LPS stimulation and has been implicated as the kinase involved in the tyrosine phosphorylation of TLR4 (13). In this study, we determined that MD-2 is also present in a complex with TLR4 and Lyn. Additionally, we observed that MD-2 could immunoprecipitate with Lyn in the absence of CD14 and TLR4 and colocalize, most likely in the ER. Furthermore, we determined that LPS-induced MD-2 tyrosine phosphorylation is strongly abolished following pretreatment with a Lyn-binding peptide inhibitor. Importantly, the Lyn-binding peptide inhibitor had no effect on the phosphorylation mutants Y22F and Y131F. Because MD-2 and Lyn seem to interact directly with one another, and MD-2 tyrosine phosphorylation is diminished following Lyn kinase inactivation, we hypothesized that similar to TLR4, Lyn kinase is involved in the tyrosine phosphorylation of MD-2. We also showed that Lyn is preassociated with MD-2 prior to LPS stimulation, yet it only associates with TLR4 after LPS stimulation. This observation raises the possibility that MD-2 delivers Lyn to the TLR4 complex to facilitate TLR4 phosphorylation by Lyn, as well as phosphorylating MD-2. Although Lyn and MD-2 seem to be preassociated, MD-2 phosphorylation does not occur until LPS/TLR4 signaling is initiated, perhaps triggering some conformational shift that allows Lyn to phosphorylate MD-2.

The mechanism by which Lyn kinase might interact and phosphorylate MD-2 is still unclear. MD-2 is a glycosylated protein that resides in the outer membrane or as a secreted form. Although there is debate about this, several investigators suggested that MD-2 plays a chaperone role and is required for proper localization of TLR4 to the surface of the (27). Lyn kinase is a myristoylated and palmitoylated protein tyrosine kinase; therefore, it is also located in the lipid rafts and in the caveolae, in the inner portion of the outer membrane, where it helps to initiate signaling events and giving it access to the signaling machinery of TLR4 (28, 29). MD-2 can also likely become tyrosine phosphorylated upon LPS stimulation during trafficking after LPS internalization. Indeed, our data showing that the endocytosis inhibitor, cytochalasin D, inhibits LPS-induced phosphorylation of MD-2 (Fig. 1B) suggests this possibility. The concept of early vesicles that are designed to fuse with each other, the relationship of receptor signaling and trafficking, and endosome signaling as it relates to generation of signal diversity and specificity is a field that has been evolving as older paradigms and dogmas are being questioned, modified, or discarded. Indeed, evidence has emerged recently that uncovered a role for the endocytic pathway in the transduction of signal dispersal and transduction of signals through the endocytic pathway and bidirectional interplay between signaling and membrane-transport networks (30, 31). Progress against old dogma in this field is further supported by recent surprising findings of ER membranes forming the phagosome membranes and ER-mediated phagocytosis (32), as well as the presence of endosomal and phagosome proteins that are phosphorylated by endogenous phagosome-associated kinases that may include tyrosine kinases (33). Additionally, a recent study found that LPS can signal through an intracellular TLR4/MD-2 pathway that did not involve recycling of surface TLR4/MD-2 (34). This new study underscores the complexity of TLR4/MD-2 signaling and that what was previously thought of as dogma (TLR4 and surface signaling) can change with data. Furthermore, this study showing that MD-2 can recognize and signal in response to LPS intracellularly makes our observation of MD-2 phosphorylation even more intriguing.

Previously, it was believed that the role of MD-2 was simply bringing together LPS and TLR4, thus providing the framework for successful signaling (7). However, our data suggest that MD-2 might play a more important role in the downstream-signaling events, given that MD-2 is a phosphoprotein that undergoes tyrosine phosphorylation upon LPS stimulation and that this event is required for optimal LPS-induced NF-κB activation. Therefore, we have defined an additional mechanism that may be involved in regulating the host innate immune response to invading pathogens and defined a novel role for MD-2, further enhancing its importance in mounting a response to LPS. Further research into the mechanism of MD-2 phosphorylation and its role in TLR4 activation is required.

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Disclosures
The authors have no financial conflicts of interest.

References


