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Separate Functional Domains of Human MD-2 Mediate Toll-Like Receptor 4-Binding and Lipopolysaccharide Responsiveness

Fabio Re* and Jack L. Strominger*†

Cellular responses to LPS are mediated by a cell surface receptor complex consisting of Toll-like receptor 4 (TLR4), MD-2, and CD14. MD-2 is a secreted protein that interacts with the extracellular portion of TLR4. Site-directed mutagenesis was used to identify the regions of human MD-2 involved in its ability to bind TLR4 and confer LPS responsiveness. A separate region of MD-2 was found to mediate each function. MD-2 binding to TLR4 was dependent on Cys95 and Cys105, which might form an intramolecular disulfide bond. Hydrophilic and charged residues surrounding this area, such as R90, K91, D100, and Y102, also contributed to the formation of the TLR4-MD-2 complex. A different region of MD-2 was found to be responsible for conferring LPS responsiveness. This region is not involved in TLR4 binding and is rich in basic and aromatic residues, several of which cooperate for LPS responsiveness and might represent a LPS binding site. Disruption of the endogenous MD-2-TLR4 complex by expression of mutant MD-2 inhibited LPS responses in primary human endothelial cells. Thus, our data indicate that MD-2 interaction with TLR4 is necessary but not sufficient for cellular response to LPS. Either of the two functional domains of MD-2 can be disrupted to impair LPS responses and therefore represent attractive targets for therapeutic interventions.x The Journal of Immunology, 2003, 171: 5272–5276.

The LPS derived from the outer membrane of Gram-negative bacteria is among the most powerful stimulators of innate immune responses. Extremely low concentrations of LPS can be sensed by immunocompetent cells, such as macrophages and dendritic cells, leading to the release of proinflammatory mediators and effector molecules.

Cellular responses to LPS are mediated by a cell surface receptor composed of at least three proteins: CD14, Toll-like receptor 4 (TLR4)1 and MD-2 (reviewed in Refs. 1 and 2). A fourth protein, the LPS-binding protein, is also required for optimal responses (3). TLR4 is one of the 10 members of the TLR family of receptors that mediates response to a wide variety of microbial products (reviewed in Refs. 4 and 5). These receptors are type I transmembrane proteins characterized by leucine-rich repeats in their extracellular portion and by the signaling Toll-IL-1R domain in their cytoplasmic region. Several groups demonstrated that TLR4 is the signaling subunit of the LPS receptor using both genetic (6–8) and biochemical approaches (9). To function as an LPS receptor, however, TLR4 must interact with an additional protein, MD-2. MD-2 is a secreted protein that is retained on the cell surface through interaction with the extracellular portion of TLR4 (9). Despite its absolute requirement for LPS signaling (9–11), it is still unclear what role MD-2 plays in LPS signaling. In MD-2-deficient mice, LPS responses are abrogated and the TLR4 molecule appears to be preferentially retained in the endoplasmic reticulum/Golgi compartment rather than on the cell surface (11). Thus, one function of MD-2 might be the correct targeting of TLR4 to the cell membrane. The existence of an anti-TLR4 mAb that recognizes TLR4 only as a complex with MD-2 (12) suggests that MD-2 could induce a conformational change in TLR4 that might be required for proper transport of TLR4 to the cell surface. However, addition of recombinant MD-2 protein to the culture medium of cell lines that express only TLR4 and do not respond to LPS render them LPS responsive (10, 13, 14), arguing that subcellular targeting of TLR4 might not be the sole function of MD-2.

MD-2 is able to form disulfide-linked multimers, adding a further level of complexity (14, 15). It is still unclear what function the MD-2 multimers might perform but since only monomeric MD-2 can bind TLR4 and confer LPS responsiveness (15), the involvement of MD-2 multimers in the signaling process seems unlikely. Several experimental approaches indicated that MD-2’s role in the LPS response is not only structural and that this molecule might directly contribute to the recognition of the LPS molecule. MD-2 is responsible for the species-specific action of LPS mimetic substances, such as Taxol (16), and of particular types of LPS (17, 18). In addition, MD-2, TLR4, and CD14 can be chemically cross-linked to the LPS molecule (19), indicating that they are in close proximity to the agonist. Finally, MD-2 can directly bind LPS in some experimental settings (13). Interestingly, a region of human MD-2 spanning aa 119–132 contains several features common to other LPS-binding proteins (20). This region is rich in basic and aromatic residues that were proposed to interact with the negatively charged and hydrophobic pattern of the LPS molecule. A synthetic peptide derived from this sequence was shown to be able to bind LPS and, remarkably, was also shown to possess antimicrobial activity (20).

Thus, although the mechanistic aspects of the interaction among TLR4, MD-2, and LPS are still obscure, the existing data would suggest the existence of two functional domains in MD-2, one responsible for TLR4 binding and another that mediates the interaction with the agonist. In this study, using site-directed mutagenesis, we report the identification of MD-2 residues that are important for each function.

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3 Abbreviations used in this paper: TLR4, Toll-like receptor 4; β-Gal, β-galactosidase; ELAM, endothelial leukocyte adhesion molecule.

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Materials and Methods

Cells

HeLa cells were grown in DMEM supplemented with 10% FCS. The HeLa-TLR4 cell line was previously described (15). Pooled HUVEC were purchased from Clonetics (Walkersville, MD). HUVEC were grown on gelatin-coated Petri dishes and maintained for <10 passages in human endothelial-serum-free medium basal growth medium (Invitrogen, San Diego, CA) supplemented with 20% FCS (Invitrogen), 10 ng/ml human recombinant epidermal growth factor (Intergen, Purchase, NY), 15 ng/ml human recombinant basic fibroblast growth factor (Intergen), and 1 μg/ml heparin (Sigma-Aldrich, St. Louis, MO).

Expression vectors

The expression vector for soluble forms of TLR4 (Flag-sTLR4) was described previously (15). MD-2-Flag-His was expressed using the vector pBOS-MD-2-Flag-His kindly provided by Dr. K. Miyake (Saga Medical School, University of Tokyo, Tokyo, Japan).

Site-directed mutagenesis

All mutations (Cys to Ala) were introduced into pBOS-MD-2-Flag-His using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s recommendations and were confirmed by sequencing. Primers sequences are available upon request.

TLR4/MD-2 complex purification by nickel resin chromatography

HeLa cells (7 × 10^6) were transfected by electroporation (250 V, 950 μF) with 30 μg of a plasmid mixture (20 μg of Flag-sTLR4 and 10 μg of MD-2-Flag-His) in Dulbecco’s PBS/1.25% DMSO. Cells were replated in 10-cm plates in FCS-containing medium to allow recovery and cell adhesion. After 12 h, plates were rinsed in PBS and 6 ml of serum-free medium 293 SFM (Invitrogen) was added. Media were collected 24–48 h later. To capture histidine-tagged protein complexes, filtered supernatants (typically 5 ml) were incubated overnight with ProBond nickel resin (Invitrogen), washed in PBS, and the resin was resuspended in Laemmli sample buffer, boiled, and analyzed by SDS-PAGE and immunoblot.

Luciferase assay

HeLa-TLR4 were transiently transfected in six-well plates using Superfect reagent (Qiagen, Valencia, CA) with 0.66 μg of CMV-enhanced green fluorescence protein (EGFP, Clontech, Palo Alto, CA) and 0.16 μg of pBOS-CD14 (kindly provided by Dr. D. Golenbock, University of Massachusetts, Worcester, MA), and 0.16 μg of CMV-β-galactosidase (β-Gal). For HUVEC transfection, cells were briefly trypsinized, washed in DMEM with 10% FCS, and resuspended in ice-cold PBS with 1.25% DMSO at a concentration of 2 × 10^5 cells/ml. Three hundred microliters of the cell suspension was electroporated with a total of 30 μg of plasmid DNA (7 μg of ELAM-luciferase, 3 μg of CMV-CD14, 1 μg of CMV-β-Gal, and 20 μg of pBOS-MD-2-Flag-His or CMV-enhanced green fluorescence protein) at 250 V, 960 μF, using a Bio-Rad (Hercules, CA) electroporator. Cells were replated in gelatin-coated six-well plates. Four hours after electroporation, cell culture medium was aspirated and cell debris were removed by PBS rinsing. Fresh complete medium was added and cells were used for experiments 36 h later. Luciferase assay was performed using Promega (Madison, WI) reagents according to the manufacturer’s recommendations. Efficiency of transfection was normalized by measuring β-Gal in cell lysates.

Results

MD-2’s domain of interaction with TLR4

We have previously developed a biochemical assay to characterize in detail the interaction between MD-2 and the extracellular portion of TLR4 (15). Using this assay, the interaction between these two proteins was shown to occur in solution and the ability of a panel of MD-2 mutants to bind TLR4 was analyzed. Mutation of two cysteines did not affect MD-2 binding to TLR4 and decreased their ability to render the cell line LPS responsive by 40–70%. Thus, Cys^95 and Cys^105 may form an intramolecular disulfide bond that creates a tertiary structure that is required for MD-2-TLR4 interaction. The analysis of the MD-2 amino acid sequence reveals that the majority of the residues that separate Cys^95 and Cys^105 are more distantly located from this region, such as K55A, K58A, R90A, R91A, Y102A (data not shown) resulted in MD-2 mutants that did not bind TLR4 were also impaired in this function with
different degrees of severity. At one extreme was the mutant Y102A that was completely incapable of conferring LPS responsiveness. At the other extreme was the mutant D100G that retained some ability to confer LPS responsiveness despite its apparent inability to bind TLR4. This last result can be explained assuming that mutation D100G might lower the affinity of MD-2 for TLR4 to a degree that makes it impossible to detect the interaction using our in vitro assay. However, a weak interaction might still occur in vivo and might be sufficient to confer some level of LPS responsiveness. Consistent with this explanation is the fact that a weak binding between TLR4 and this MD-2 mutant is occasionally detected in our assay (data not shown). Also in agreement with this explanation is the observation that addition of the mutant protein D100G to the culture supernatant of the HeLa-TLR4 cell line cannot render it LPS responsive as the wild-type protein is able to do (data not shown).

These results thus identify the region of MD-2 that is responsible for its interaction with TLR4. They also demonstrate that to confer LPS responsiveness MD-2 must bind to TLR4.

**MD-2’s domain responsible for LPS responsiveness**

It has been suggested that a region of MD-2 (aa119–132) rich in basic and aromatic residues might be able to interact with the LPS molecule (20). To test whether this region of MD-2 is indeed the putative LPS binding site, several of the basic and aromatic residues were mutagenized, either singularly or in different combinations, and the ability of the mutant proteins to bind TLR4 and confer LPS responsiveness was examined. Single mutations (data not shown) or double mutations in any of these residues did not affect the ability of MD-2 to bind TLR4 (Fig. 3A). Even a quadruple mutant completely retained the ability to form a complex with TLR4. However, the ability of these mutants to render HeLa-TLR4-LPS responsive was progressively affected as the mutations accumulate (Fig. 3B). As expected for mutations that would decrease the affinity of MD-2 for the LPS molecule, the effect on LPS responsiveness was more pronounced as the agonist concentration was lowered. Thus, response to maximal doses of LPS was reduced 80% by mutation F121G-K122A, 20% by mutation K125A-F126A, and 83% by mutation Y131G-K132A. However, the response of these mutants was totally impaired at suboptimal LPS concentrations that are nevertheless still able to elicit a powerful response through the wild-type MD-2. The combination of the four mutations F121G-K122A-Y131G-K132A completely disrupted the response to LPS even at the highest LPS doses. Thus, although still able to bind TLR4 to the same extent as the wild-type MD-2, the mutant F121G-K122A-Y131G-K132A is totally LPS unresponsive. These experiments indicate that this region of MD-2 contains several residues that might cooperate to create the LPS binding site. These results also suggest that MD-2 interaction with TLR4 is necessary but not sufficient for proper TLR4 responses to LPS.

**Disruption of the endogenous MD-2-TLR4 complex by mutant MD-2 inhibit LPS responses in HUVEC**

The complex TLR4/MD-2 is believed to form in the endoplasmic reticulum/Golgi compartment. The hypothesis that some of the MD-2 mutants might compete with the endogenous MD-2 protein for binding to TLR4, and therefore act as dominant negatives and...
Cys 95 and Cys 105. A putative disulphide bond in MD-2 binding to TLR4 is necessary but not sufficient for LPS responsiveness. Two separate functional domains in MD-2, one required for interaction with TLR4 and LPS responsiveness has been analyzed. The ability of a panel of MD-2 mutants to bind TLR4 and confer LPS responsiveness was also found to play a similar role in mouse MD-2. Specifically, mutation of Cys 95, Tyr 102, and Cys 105 disrupted the ability to form the TLR4-MD2 complex also in mouse cells. Surprisingly, mutation of Cys 37 and Cys 140 disrupted the mouse TLR4-MD2 complex but not the human’s. Although highly homologous, human and murine TLR4 and MD-2 show species specificity (16–18). It is possible that the interaction between the two proteins has different structural requirements in the two species. Another possible reason for the discrepancies between our study and that of Kawasaki et al. (23) is probably due to the different methods used to study the TLR4-MD2 complex formation: our study relies on an in vitro biochemical assay to detect a direct interaction between the two proteins. Kawasaki et al. (23) monitored the increase in the reactivity to an anti-TLR4 Ab that recognizes TLR4 only when complexed to MD-2 rather than analyzing a direct TLR4-MD2 interaction.

The residues responsible for LPS responsiveness also seem to be scattered throughout the mouse MD-2 molecule rather than being concentrated in one discreet region as our study indicates to occur in the human protein. The study of Kawasaki et al. (23), although more exhaustive than ours, analyzed only single mutations. In contrast, our study showed that single mutations are not sufficient to completely disrupt LPS responsiveness and the putative LPS binding site was identified only by analyzing multiple mutations. Interestingly, some of the mouse MD-2 residues that affected LPS responsiveness did not affect responses to Taxol, suggesting that the two ligands interact with different regions of the mouse protein. This observation would suggest that also in human MD-2 additional domains might exist that mediate responsiveness to other TLR4 agonists, such as heat shock protein 60 (24), respiratory syncytial virus protein F (25), and fibronectin extradomain A (26).

The identification of the regions of MD-2 involved in the interaction with TLR4 and LPS is a prerequisite for the design of specific inhibitors of LPS actions. Our study identifies two distinct strategies that can be used to block LPS responses: disruption of the MD-2-TLR4 complex or inhibition of MD-2 interaction with LPS.

Discussions

The ability of a panel of MD-2 mutants to bind TLR4 and confer LPS responsiveness has been analyzed. Two separate functional domains in MD-2, one required for interaction with TLR4 and a distinct one responsible for LPS responsiveness, were identified. MD-2 binding to TLR4 is necessary but not sufficient for LPS responsiveness.

The MD-2’s domain of interaction with TLR4 is dependent on Cys 95 and Cys 105. A putative disulphide bond between these residues would create a tertiary structure that might be exposed to the aqueous phase and available for the interaction with TLR4. Several residues comprised or surrounding this area, such as D100, Y102, R90, and K91, are important for interaction with TLR4.

The ability to confer LPS responsiveness maps to a different portion of MD-2 that is not involved in TLR4 binding. This area is rich in basic and aromatic residues. Although single mutations in various residues contained in this area affect LPS responsiveness to different degrees, the combination of multiple mutations progressively impair this function with mutant F121G-K122A-Y131G-K132A being completely unresponsive while still able to bind TLR4. Thus, MD-2 interaction with TLR4 is necessary but not sufficient for TLR4-mediated responses to LPS.

A similar conclusion was reached by a recently published study in which a saturating alanine scanning mutagenesis of mouse MD-2 was conducted (23). The mutant proteins were tested for their ability to increase reactivity to an anti-TLR4 Ab that recognizes TLR4 only when complexed with MD-2 and for conferring responsiveness to LPS and Taxol, a substance that has LPS mimetic activities in mouse but not human cells. Although this study failed to identify any particular region in mouse MD-2 important for each function, several of the residues that were found in the present study to be involved in the ability of human MD-2 to bind TLR4 and confer LPS responsiveness were also found to play a similar role in mouse MD-2. Specifically, mutation of Cys 95, Tyr 102, and Cys 105 disrupted the ability to form the TLR4-MD2 complex also in mouse cells. Surprisingly, mutation of Cys 37 and Cys 140 disrupted the mouse TLR4-MD2 complex but not the human’s. Although highly homologous, human and murine TLR4 and MD-2 show species specificity (16–18). It is possible that the interaction between the two proteins has different structural requirements in the two species. Another possible reason for the discrepancies between our study and that of Kawasaki et al. (23) is probably due to the different methods used to study the TLR4-MD2 complex formation: our study relies on an in vitro biochemical assay to detect a direct interaction between the two proteins. Kawasaki et al. (23) monitored the increase in the reactivity to an anti-TLR4 Ab that recognizes TLR4 only when complexed to MD-2 rather than analyzing a direct TLR4-MD2 interaction.

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References

MD-2’s DOMAINS FOR TLR4 BINDING AND LPS RESPONSIVENESS


