Structural Model of MD-2 and Functional Role of Its Basic Amino Acid Clusters Involved in Cellular Lipopolysaccharide Recognition*

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The receptor complex resulting from association of MD-2 and the ectodomain of Toll-like receptor 4 (TLR4) mediates lipopolysaccharide (LPS) signal transduction across the cell membrane. We prepared a tertiary structure model of MD-2, based on the known structures of homologous lipid-binding proteins. Analysis of circular dichroic spectra of purified bacterially expressed MD-2 indicates high content of β-type secondary structure, in agreement with the structural model. Bacterially expressed MD-2 was able to confer LPS responsiveness to cells expressing TLR4 despite lacking glycosylation. We identified several clusters of basic residues on the surface of MD-2. Mutation of either one of two clusters encompassing the residues Lys86-Arg90-Lys91 and Lys125-Lys125 significantly decreased the signal transduction of the respective MD-2 mutants either upon co-expression with TLR4 or upon addition as soluble protein into the supernatant of cells overexpressing TLR4. These basic clusters lie at the edge of the β-sheet sandwich, which in cholesterol-binding protein connected to Niemann-Pick disease C2 (NPC2), dust mite allergen Der p2, and ganglioside GM2-activator protein form a hydrophobic pocket. In contrast, mutation of another basic cluster composed of Arg69–Lys72, which according to the model lies further apart from the hydrophobic pocket only weakly decreased MD-2 activity. Furthermore, addition of the peptide, comprising the surface loop between Cys85 and Cys88, predicted by model, particularly in oxidized form, decreased LPS-induced production of tumor necrosis factor α and interleukin-8 upon application to monocytes and fibroblasts, respectively, supporting its involvement in LPS signaling. Our structural model of MD-2 is corroborated by biochemical analysis and contributes to the unraveling of molecular interactions in LPS recognition.

Response of mammalian cells to bacterial infection is mediated by receptors that recognize the presence of pathogen-associated molecular patterns in the extracellular space and signal their presence into the cell interior through specific transmembrane Toll-like receptors (TLR)

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(Diversity of TLRs enables fine-tuning of host response to a wide variety of different microbes such as bacteria and fungi. In mammals, signaling of bacterial lipopolysaccharide, a major component of the cell wall of Gram-negative bacteria, proceeds through TLR4 (2). Despite reports of close proximity between TLR4 and LPS (3), TLR4 might not bind LPS directly but requires the presence of an extracellular adaptor protein MD-2, which is bound to its ectodomain (4, 5). MD-2 associates with TLR4 within the endoplasmic reticulum already and is responsible for its proper glycosylation and trafficking to the cellular surface (5, 6). The TLR4-MD-2-LPS complex recycles rapidly between cell surface and Golgi (7). Mature MD-2 is glycosylated, encompasses 142 amino acid residues, and directly binds LPS with high affinity (8, 9). Addition of soluble MD-2 to principally LPS-unresponsive cells overexpressing TLR4 renders them responsive to LPS challenge (4). Binding of LPS to MD-2-TLR4 complexes induced formation of large receptor clusters on the cell surface and crosslinking of either TLR4 or MD-2 with surface bound antibodies lead to cell activation (10). Murine but not human MD-2 and TLR4 confers responsiveness to taxol (11). A systematic alanine scanning mutagenesis study of murine MD-2 identified specific residues important for interaction of LPS and taxol with murine MD-2 and TLR4 (12). MD-2 forms disulfide-linked dimers, as well as larger oligomers, which can be disrupted by disulfide reduction (13). The relevance of those oligomers for LPS signaling is not yet clear (14, 15). A point mutation (C95Y) in MD-2 of LPS nonresponder mice (16) and targeted point mutations have led to identification of regions that are important for interactions with TLR4 (17) or affect interaction with LPS (10, 17), some of which have been identified previously by the application of MD-2 peptide fragments (18). However, only information about the tertiary structure of MD-2 would enable to put these results into a broader perspective and comprehensively elucidate the molecular interactions involved in LPS recognition.

The only tertiary structure of the LPS-protein complex has been determined for the bacterial iron uptake receptor PhuA (19). This structure initiated the proposition of an LPS-binding motif, defined by the geometric arrangement of basic residues, which has also been identified on several LPS-binding proteins (20), as well as on other proteins. The proposal of an LPS-binding motif was based solely on electrostatic interactions.

1 The abbreviations used are: TLR, Toll-like receptor; bMD-2, MD-2 expressed in bacterial cells; LPS, lipopolysaccharide; NPC2, protein deficient in Niemann-Pick type C2 disease; Der p2, dust mite allergen; GM2-AP, ganglioside GM2 activator protein; GM2, II3NeuAc-GgOse,Cer; HEK, human embryonic kidney; FCS, fetal calf serum; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; wt, wild type.

2 R. Jerala, unpublished observation.
which are certainly not sufficient since the importance of the number of acyl chains in LPS recognition has been well recognized (21). The structural homology of the LPS recognition motif of MD-2 is more likely to be found among the complexes of soluble proteins with glycolipids such as gangliosides, for example, rather than among the integral membrane proteins such as FhuA. The structure of GM2 ganglioside activating protein (GM2-AP) has been determined both in free form and in complex with ganglioside ligands (22, 23). In the case of GM2-AP the glycolipid binds into the apolar pocket formed between the β-sheets and involves significant conformational changes between the closed and open conformation of the binding pocket. Structures of other members of this protein superfamily have been determined which show a lipid-binding pocket expansion after binding of ligands such as cholesterol to NPC2 protein (24) or an unidentified hydrophobic ligand to the mite allergen protein Der p2 (25).

The aim of our work was to provide a model of the tertiary structure of MD-2 and assess its reliability by relating it to recently reported characteristics of MD-2 and our experimental results. We have prepared a tertiary structure model of MD-2, which allowed delineation of functional molecular epitopes essential for LPS signaling.

MATERIALS AND METHODS

Peptides—A peptide MD2s (Ac-CHRGSDDDYTFSC-NH2) was synthesized by the W. M. Keck facility at Yale University using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) method. The peptide was oxidized by overnight incubation in 50 mM sodium carbonate buffer, pH 8.5. The oxidized and reduced form of the peptide were separated and purified by high performance liquid chromatography. The concentration of the free SH group was determined by using 5,5'-dithiobis(2-nitrobenzoic acid) reaction, which binds to free SH groups (26).

Cell Lines—Human embryonic kidney (HEK) 293 cells (ATCC number CRL-1573) and THP-1 cells (ATCC number TIB-202) were used. HEK293 cells were cultured as adherent monolayer at 37 °C, 8% CO2 and 95% humidity in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) FCS (PAA, Laboratories) and 1% (v/v) penicillin-streptomycin (Invitrogen). THP-1 cells were grown in suspension culture in RPMI (Invitrogen) supplemented with 10% (v/v) FCS (PAA, Laboratories), 1% (v/v) l-glutamine (Invitrogen), and 1% (v/v) penicillin-streptomycin (Invitrogen). For culture, 10-fold dilutions in fresh medium were prepared from cells grown in culture flasks for 3 days.

Homology Modeling of MD-2—Search for homologues of human MD-2 in the GeneBank™ data base of translated nucleotide sequences was performed using BLAST or PSI-BLAST (27). Fold recognition was performed and evaluated with metaserver 3D-Fury, which combines 13-fold prediction recognizer algorithms and has been one of the most successful predictors in the recent CASP5 protein structure prediction assessment (28). Sequential alignment for homology modeling was based on the sequence threading identified by the Fuego2 algorithm (29).

Tertiary structures of bovine cholesterol-binding protein connected to Niemann-Pick disease (NPC2) (Protein Data Bank code 1NEP) (24) and Der p2 mite allergen structures (Protein Data Bank codes 1A9V and 1KTJ (25, 30)) were used as templates for comparative modeling. The program MODELLER, version 6.2 (31) was used to generate 100 tertiary structure models using constrained disulfide bonds in the same topology as in tertiary structures of ML proteins. Structures were refined by MD annealing. The structure prediction of loop 38–51 was additionally optimized by application of the ModLoop program (32) in the context of complete structure, which further improved the energy function of the former model. Tertiary structure models were ranked by the “Modeler’s objective function” and evaluated with Procheck (33) and subsequently by Verify3D (34) to identify the regions of highest and lowest quality of the model.

Preparation of Recombinant MD-2 and Determination of Its Secondary Structure—The coding sequence of mature human MD-2, provided by Dr. Miyake, was amplified by PCR (primers AGCTCTCATTCGCCGA-GAACGACTTTGTTGCTTC and AGCTTCGAGCTAAAGTTTAGAAGTGGTTGAGG) introduced through NcoI and XhoI sites into the pET14b cloning vector and protein expressed in BL21(DE3) pLysS bacterial cells (Novagen) (35). Recombinant protein was produced in form of inclusion bodies, which were washed with 0.1% B-PER® Bacterial Protein Extraction Reagent (Pierce). The resulting protein pellet was solubilized in 6 M guanidinium hydrochloride, 20 mM sodium phosphate, pH 5.0, and applied to RP SDB monolithic disks (BIA Separations, Ljubljana, Slovenia). Proteins were separated and eluted upon application of acetonitrile and 0.05% trifluoroacetic acid at a gradient of 5–95% acetonitrile to the column. After solvent evaporation, eluted proteins were dissolved in 10 mM sodium phosphate buffer at pH 7.0. SDS-PAGE confirmed homogeneity of the protein. Circular dichroic spectra of recombinant MD-2 at a concentration of 0.18 mg/ml dissolved in water were measured on an Aviv spectropolarimeter 62A (Aviv Associates) using quartz cuvettes with a path length of 0.1 cm for the far-UV region (190–260 nm). For the circular dichroism data analysis, the circular dichroism was calculated as the mean residue ellipticity θ222 (degree·cm2·dmol−1) using a mean molar residue weight of 115 for calculation.

Site-directed Mutagenesis—Specific point mutations were introduced into human MD-2 using site-directed mutagenesis. A site-directed mut-
tagenesis Kit (Stratagene, La Jolla, CA) was used, and the reaction was performed according to manufacturer’s instructions. The sequences of primers used for PCR were as follows: MD2/70, 5'-CACATTTTTCTACA-TTCCAAAGGGCAGATTAGCGGCAATATTCTATATTTCACTCTATATATAA-CTG-3' and 5'-AGTTTAAATAGGATTTAATATGTTGACAAATGCTATTCCCTTG-3'; MD2/90, 5'-CACATGAGTTCCACAGCCGGAGTTAGGAGTTAATGTTGACAAATGCTATTCCCTTG-3' and 5'-AGTTTAAATAGGATTTAATATGTTGACAAATGCTATTCCCTTG-3'; MD2/100, 5'-CTCCTCAAGGGAAATACCTTTCTCCTCCGGGAAAAATACTAAAAATGTGGTG-3' and 5'-CAGAGTTATATAGAGATTGAAATATAATTGCGCTAAATGCTATTCCCTTG-3'. The complete sequences of MD-2 mutants were controlled by sequencing.

Preparation of Cellular Extracts—HEK293 cells were detached from cell culture plates with a diameter of 9 cm in 5 ml of phosphate-buffered saline and harvested by spinning for 7 min at 4 °C and 1200 rpm. Cell pellets were resuspended in 40–100 μl of lysis buffer (36), transferred to Eppendorf tubes, and incubated on ice for 20 min. Cell debris was removed from the suspension by spinning twice for 20 min at 4 °C and 13,000 rpm.

Preparation of MD-2 Conditioned Supernatants—1 × 10⁶ cells of cell clones stably expressing MD-2 mutants were seeded in a 15-cm dish in 10% FCS and grown overnight. Medium was exchanged for 2% FCS medium, constitutive reporter plasmid, and 2.5 ng of TLR4 expression vector. Various dilutions of bMD-2 (stock concentration was 30 μM) were incubated with 100 ng/ml LPS prior to stimulation of TLR4 transfected HEK293 cells. Conditioned supernatant from MD-2 producing HEK293 cells with estimated concentration of MD-2 of 1 μg/ml was used as a positive control.

Human IL-8 and TNFα ELISA—IL-8 and TNFα release from HEK293 and human monocytic THP-1 cells were determined in 96-well plates using ELISA kits purchased from R & D Systems (Minneapolis, MN). 1 × 10⁶ cells were seeded per well of a 96-well plate. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate at a concentration of 50 ng/ml for 24 h. MD-2 peptides were added to a final concentration of 2 μg/ml. Supernatant from HEK293 cells expressing bMD-2 without or with added LPS was collected after 16 h of stimulation.

Luciferase Reporter Assay—HEK293 cells were transiently transfected with NF-κB-dependent and constitutive reporter plasmids, as well as 2.5 ng of TLR4 and MD-2 expression vectors. 24 h after transfection start cells were challenged with agonists for 16 h. For analysis of MD-2 peptide function, peptides were added 60 min prior to stimulation at a final concentration of 25 μg/ml. The cells were lysed in reporter lysis buffer (Promega, Madison, WI) and lysates analyzed for reporter gene activities. β-Galactosidase activities were analyzed and used for normalization.

For measuring the activity of bacterially expressed MD-2, HEK293 cells were transiently transfected with NF-κB-dependent reporter plasmid, constitutive reporter plasmid, and 2.5 ng of TLR4 expression vector. Various dilutions of bMD-2 (stock concentration was 30 μM) were incubated with 100 ng/ml LPS prior to stimulation of TLR4 transfected HEK293 cells. Conditioned supernatant from MD-2 producing HEK293 cells with estimated concentration of MD-2 of 1 μg/ml was used as a positive control.

Flow Cytometry Analysis—HEK293 cells were seeded and transiently transfected. 56 h later, cells were harvested in 5 ml of cold phosphate-buffered saline and washed. An aliquot of 5 × 10⁶ cells was stained in a volume of 60 μl. Liquid was removed, and primary antibodies (αFLAG, αMyc) were applied at a concentration of 8 μg/ml and incubated for 20 min on ice. Cells were washed twice. Secondary peroxidase-coupled antibodies were added to a final concentration of 2 μg/ml. Samples were incubated for 20 min on ice and washed twice again. Fluorescence measurement was carried out on a fluorescence-activated cell sorter Calibur flow cytometer, and data were processed using the CellQuest software (BD Bioscience, Heidelberg, Germany).

**Fig. 2. Schematic representation of structural model of MD-2. Left panel,** schematic representation of MD-2 backbone with highlighted disulfide cysteine residues (gold spheres) and Cys⁵⁵–Cys⁸⁵ loop (MD2S peptide, red). **Right panel,** surface representation of point mutants tested in this work. The MD2S loop is shown in red, mutations are shown by van der Waals radii: MD2/70 (blue), MD2/90 (orange), MD2/120 (green).

**Fig. 3. Properties of bacterially expressed MD-2; biological activity and high content of β-type secondary structure of MD-2. A, 15% SDS-PAGE of bMD-2 under reducing and nonreducing conditions. Left lane, molecular mass standards shown in kilodaltons; middle lane, reduced bMD-2; right lane, nonreduced bMD-2. B, activation of TLR4 transfected HEK293 cells monitored by luciferase activity in the presence of dilutions of bMD-2 (30 μM) preincubated with 100 ng/ml LPS in comparison with LPS, supernatant of HEK293 cells producing MD-2, or bMD-2 without added LPS. C, far-UV CD spectrum of purified bMD-2, determined in water at 0.18 mg/ml.**
concentration of 25 µg/ml. 60 min later, ultrapure LPS from *Salmonella minnesota* Re595 (List, Campbell, CA) was added. Supernatants were removed 16 h (HEK293) or 4 h (THP-1) after stimulation and analyzed by ELISA. Duplicate samples were prepared and analyzed.

**RESULTS**

**Fold Recognition and Model of Tertiary Structure of MD-2**

Search through the GeneBank™ data base for amino acid sequences similar to human MD-2 revealed nine additional MD-2/MD-1 family members. The more sensitive fold recognition approach, which is able to detect structurally compatible sequences (28), recognized similarity to the members of a family of lipid-binding proteins (ML superfamily (37)) also including a number of proteins for which tertiary structures have been determined. Consensus fold recognition by the 3D-Jury metaserver (28) identified compatibility of the MD-2 sequence with tertiary structures of members of the early Ig-like family of protein from the SCOP (structural classification of proteins (38)) data base and ranked them on the first 11 positions of the resulting list. The secondary structure of MD-2 was predicted using a consensus of secondary prediction algorithms implemented in the PredictProtein server (39). The majority of MD-2 residues were predicted to be in the extended (β) structure (55%) with a minimal content of helical structure (5%). The structure fold most compatible with human as well as murine MD-2 sequence was Der p2 protein (Protein Data Bank code 1A9V) (30), which contains 38% of sequence similarity and 16% identity to human MD-2, followed by NPC2 (Protein Data Bank code 1NEP) (24) with 26% of similarity and 12% of identity. The predicted secondary structure of MD-2 agrees with secondary structure content of those two tertiary structures. Application of the Fugue2 fold recognition algorithm (29) estimated that the probability of Der p2 and human MD-2 having the same structural fold is >95%. Comparative structure modeling using program Modeler (31) was based on the alignment from fold recognition. Within the alignment of MD-2 homologues along with NPC2 and Der p2, locations of six of the cysteine residues present in all of them were congruous suggesting conservation of disulfide-bonding topology (Fig. 1). The only unpaired cysteine in MD-2 having no counterpart in NPC2 or Der p2 is Cys, which is absent in MD-1 protein family. Our structural model of MD-2 contains the following features. MD-2 has the sandwich-type fold composed of seven strands arranged in two layers of antiparallel β-sheets. In comparison to NPC2 and Der p2 MD-2 contains two insertions composed of eight and five residues, which were modeled as expansions of surface loops already present in Der p2 and NPC2 (Fig. 2). Both loops are flanked by disulfides, which are conserved in the tertiary structures of ML proteins. The conformation of surface loops in the
model should be rather regarded as undefined in our model, since the reliability of theoretical modeling of such insertions is generally low, which is also reflected by the fact that Verfy3D score was the lowest in the eight-residue insertion loop (residues 39–51). The global features of our model of MD-2 fold, however, are most likely correct as they were confirmed by the results of our functional analysis based on the model predictions as follows.

Secondary Structure of Recombinant MD-2—Purified and refolded bMD-2 also formed dimers and higher order aggregates linked by disulfides (Fig. 3A) as MD-2 produced by eucaryotic cells (13). bMD-2 conferred LPS reactivity to HEK293 cells transfected with TLR4 at concentrations below 30 nM, which is comparable with the specific activity of MD-2 from eucaryotic cell supernatant (Fig. 3B). Addition of MD-2 slightly increased the base-line activation of TLR4 transfected HEK293 cells as reported before from supernatant of eucaryotic cells (40), but maximal response was achieved by stimulation with LPS. UV circular dichroic (CD) spectrum of refolded MD-2 is typical of a protein rich in β structure (Fig. 3C). Maximum of intrinsic fluorescence emission of MD-2, which contains a single tryptophan residue (Trp23), was at 348 nm (data not shown), indicating high solvent exposure of this residue, which is also in agreement with tertiary structure model (predicted 24% exposure to solvent).

Clusters of Basic Amino Acids Are Not Involved in Association to TLR4—Analysis of the electrostatic potential of MD-2 model revealed that most of the protein, with marked exception of the region in the loop Cys335–Cys355 has a strongly positive (basic) potential (data not shown). According to the model, there are several clusters of basic residues particularly at the rim of the β-sheet sandwich. We have mutated three clusters of basic residues by alanine replacement selected to support the structural model and hypothesis on MD-2 interaction with TLR4. MD2/90 (R69A, K72A, K89A, R90A, K91A), MD2/120 (K125A, K128A), All mutant proteins were successfully expressed and presented at the cell surface (Figs. 4 and 5). In the presence of TLR4 expression surface presentation of wild type and MD-2 mutants was increased except in case of MD-2/90 mutants. Immunoprecipitation indicated that all MD-2 mutant proteins were bound to TLR4 upon overexpression, although MD2/90 and MD2/70–90 associated with the 130-kDa, fully glycosylated form of TLR4 (41) to a significantly lesser degree than MD2/70, MD2/120, and wtMD-2, while association to the 110-kDa form was equal (Fig. 5).

Clusters of Basic Residues of MD-2 Involved in LPS Signaling—HEK293 cells expressing TLR4 were transfected with MD-2 expression constructs. The largest relative decrease in MD-2-mediated cell activation as compared with wild-type MD-2 was observed upon its substitution by mutant constructs MD2/90 and MD2/120 (Fig. 6), which, according to our model, are positioned at the edge of the hydrophobic pocket, while the mutant cluster of MD2/70 is located further away. Application of MD2/70 resulted in a significantly lesser impairment of cell activation as compared with cell activation by mutants MD2/90 and MD2/120 (Fig. 6). Double mutant MD2/70–90 also conferred weak reporter gene activation and IL-8 release even if the LPS concentration was increased to 1 μg/ml (data not shown). These results were supplemented through the addition of conditioned supernatants containing equal amounts of soluble MD-2 mutants to cells readily overexpressing either TLR4 only or the complex of TLR4/wtMD-2. Only the mutant construct MD2/70 imparted high LPS responsiveness, MD2/120 mutant mediated only weak cell activation, while mutants MD2/90 and MD2/70–90 did not confer detectable LPS responsiveness (Fig. 6). Application of supernatant containing any of the overexpressed MD-2 mutant constructs to cells overexpressing both TLR4 and wild-type
MD-2 did not influence responsiveness to LPS significantly (Fig. 7) indicating high stability of the preformed TLR4-MD-2 complex.

Inhibition of LPS Signaling by Disulfide-linked Peptide Fragment of MD-2—The peptide sequence representing the insertion region 95–105 in MD-2 in comparison with NPC2 or Der p2 structures was chosen for peptide synthesis. This peptide (MD2S) was applied to cells expressing TLR4 and MD-2. MD2S does not encompass the proposed LPS-binding motif, did not bind or neutralize LPS in vitro, and did not activate cells in the absence of LPS (data not shown). When peptide was added 60 min prior to LPS stimulation, a significant inhibition of NF-κB-dependent luciferase activity was observed in the case of peptide MD2S and even higher for its oxidized form (MD2S-oxidized) (Fig. 8). Cellular activation decreased to 40–60% as compared with cells treated with control peptide and stimulated with LPS upon application of MD2S. Oxidized peptide MD2S, which interfered with LPS signaling, to the largest extent appeared to be equally potent in experiments in which peptide and LPS were co-incubated prior to addition to cells or separately (data not shown). In human macrophage-like THP-1 cells, the reduced form of MD2S peptide did not significantly decrease TNFα secretion, while application of the oxidized peptide again decreased the level of TNFα secretion by 40% (Fig. 8C).

**DISCUSSION**

The quest for identification of molecular interactions that govern the recognition of LPS focused on Lbp, CD14, and MD-2/TLR4 as the specific LPS recognition molecules acting in an ordered endotoxin recognition cascade (9). Interaction of proteins and peptides with LPS structurally characterized up to now involved at least two clusters of basic residues separated by a distance matching the separation between the phosphate groups of the lipid A moiety, as well as solvent exposed hydrophobic residues (20, 42, 43). The effect of basic residues of MD-2 for LPS binding has been analyzed before by using either synthetic peptide fragments of MD-2 or point mutations (10, 17, 18). The cumulative effect of decreasing the number of basic residues has been demonstrated for the residues, which formed the largest continuous stretch of basic and hydrophobic residues (Phe121–Lys132) (17). The effect was even more pronounced in charge reversal mutants of Lys128 and Lys132 (10). The effect of knocking out several basic residues can be explained through the disturbance of the positive electrostatic potential, which may steer LPS toward the binding site. The electrostatic potential of MD-2 according to our model is the highest at the region, which roughly coincides with the lipid-binding sites of NPC2, Der p2, or GM2-AP. Residues with negative charge, on the other hand, are concentrated at the Cys95–Cys105 loop in human MD-2 as well as in its orthologues. Effects of eliminating different basic clusters of
Cys95 and Cys105 are the two cysteine residues whose mutation knock-in study of MD-2 cysteine residues demonstrated that binding to TLR4 (17) resulted in a decrease in LPS responsiveness and conserved surface interaction with TLR4 (reactivity with monoclonal antibody MTS510) are shown in gray (labeled residues in Fig. 3A in original publication (12)), and residues with decreased reactivity to MTS510 (interaction with TLR4) are shown in black (labeled residues in Fig. 3C in original publication (12)).

Fig. 9. Segregation of residues responsible for interaction with TLR4 and LPS signaling in tertiary structure model. Mutants of murine MD-2 from alanine scanning (12) are mapped on the backbone of the structural model of MD-2 in stereo representation. Residues with decreased LPS responsiveness and conserved surface interaction with TLR4 (reactivity with monoclonal antibody MTS510) are shown in gray (labeled residues in Fig. 3A in original publication (12)), and residues with decreased reactivity to MTS510 (interaction with TLR4) are shown in black (labeled residues in Fig. 3C in original publication (12)).

MD-2 compared in this work range from large, for mutations located in close proximity to the hydrophobic pocket (MD2/90 and MD2/120), to minor, for sites located at the opposite side of the molecule (MD2/70), indicating different functional roles based on their specific location in the structure. Mutation MD2/120 comprises sites 125 and 128, included in synthetic peptide Phe119–Lys132 (18) and mutagenesis studies (10, 17)). As discussed above, two phosphate-binding sites are expected on the LPS receptor, since both phosphate moieties of the lipid A are necessary for strong endotoxic activity, similar to what is shown in FhuA-LPS (19) and polymyxin B-LPS complex structures (42). Separation of 13–25 Å between clusters MD2/70 and MD2/120 matches the distance of 15 Å between phosphate groups of the lipid A moiety. Besides the electrostatic interactions recognition of LPS by MD-2 undoubtedly also involves additional interactions such as hydrophobic, van der Waals, and hydrogen bonding, which are necessary to achieve the low experimentally determined MD-2-LPS affinity (8). Mutations MD2/90 and MD2/120, as well as mutations of Lys128 and Lys132 (10), disturb the area of high electrostatic potential in the model (data not shown), which can affect steering of ligand to the binding site. However, MD2/90, which is located close to the C95-C105 loop, probably plays a role also in MD-2-TLR4 interaction.

It is not yet clear how or if the disulfide dimers or oligomers of MD-2 are involved in pattern recognition and signaling. According to our model, the single nonpaired cysteine of MD-2 (Cys133) is buried within the molecule, while all three disulfide bridges are exposed to the solvent (between 5 and 31%) and could probably easily be reduced (15). Mutation of Cys133 had the least effect on MD-2 activity (15, 12), which is consistent with our model. Cys95 within the short surface loop has been identified as very important for the MD-2 function, since its mutation to tyrosine abolished the activity (16) as well as its binding to TLR4 (17). A systematic mutation based knock-out/knock-in study of MD-2 cysteine residues demonstrated that Cys95 and Cys105 are the two cysteine residues whose mutation decreased cell activation to less than 20% as compared with wild-type MD-2, while up to 45% activity was preserved in the case where they were the only remaining cysteines in an MD-2 construct (15).

Residues in the short surface loop, which contains a stretch of acidic residues, have been shown to be involved in interaction with TLR4 (17). In addition, the affinity of the TLR4-MD-2 complex for LPS has been reported to be higher than the affinity of the isolated MD-2 (44). The short Cys95–Cys105 loop is positioned near the edge of the proposed hydrophobic binding pocket. Tertiary structure of GM2-AP, whose ligand ganglioside GM2 bears considerable similarity to LPS, contains a similar disulfide bridge-constrained but larger loop positioned like a flap above the ligand-binding pocket (22, 23). Our model suggests that interaction of MD-2 with TLR4 involves the Cys95–Cys105 loop and results in formation of a new environment of the LPS-binding site by either providing additional interacting sites by the residues of TLR4 or through the conformational change imparted on MD-2 by TLR4 binding.

Two N-glycosylation sites have been identified in MD-2, namely Asn26 and Asn114 (6, 45). Removal of glycosylation was reported to decrease the response to LPS although MD-2 was still able to bind LPS. Elimination of glycosylation at position 26 resulted in a larger decrease in cell activation than at position 114, which is consistent with localization of Asn26 close to the Cys95–Cys105 loop and Asn114 on the opposite face of MD-2. Carbohydrate moieties might nevertheless be involved in interactions with TLR4, although alanine mutants of the Asn26 and Asn114 residues resulted in only marginal decrease in LPS responsiveness (12), which is in line with our finding of a capacity of bacterially expressed MD-2 to confer LPS response HEK293 cells overexpressing TLR4.

Our results strongly support the three-dimensional model of MD-2 presented herein at least in terms of general folding pattern and topological positioning of functional residues. Mutants affecting LPS signaling and those decreasing regular surface presentation of MD2/TLR4 complex identified in alanine scanning of murine MD-2 (12) are clustered in two sepa-
rate regions of the molecule in our model (Fig. 9). Surface presentation of a specific epitope indicating interaction with TLR4 was affected by mutations in the region of MD-2 close to the Cys\(^{95}\)-Cys\(^{105}\) loop, while the residues involved in LPS or taxol responsiveness were clustered together at the opposite side of MD-2, lining the interior of the hydrophobic pocket (Fig. 9). In the case of cholesterol-binding protein NPC2 structural analysis showed that the existing cavity of the protein is too small to accommodate the lipid and therefore has to expand (24), which is probably the case also for MD-2. Cholesterol-binding proteins, GM2-AP and NPC2, have similar fold as MD-2. Similarity between cholesterol- and LPS-binding proteins has been observed before for LPS-binding proteins BPI and LBP sharing the same protein fold with cholesteryl ester transfer protein (46). It is interesting that the structural equivalent of the complex between MD-2 and the ectodomain of TLR4 fused into a single chain protein has been described in internalin from \(L\)isteria\ monocytogenes, which is composed of an ML domain fused to a leucine-rich repeat domain (47).

The aim of our study was identification of functional sites of MD-2 important for LPS signaling based on the model of tertiary structure. It is reassuring that alanine scanning mutagenesis analysis showed that the existing cavity of the protein is too small to accommodate the ligand and therefore has to expand (24), which is probably the case also for MD-2. Cholesterol-binding proteins, GM2-AP and NPC2, have similar fold as MD-2. Similarity between cholesterol- and LPS-binding proteins has been observed before for LPS-binding proteins BPI and LBP sharing the same protein fold with cholesteryl ester transfer protein (46). It is interesting that the structural equivalent of the complex between MD-2 and the ectodomain of TLR4 fused into a single chain protein has been described in internalin from \(L\)isteria\ monocytogenes, which is composed of an ML domain fused to a leucine-rich repeat domain (47).

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