Identification of Mouse MD-2 Residues Important for Forming the Cell Surface TLR4-MD-2 Complex Recognized by Anti-TLR4-MD-2 Antibodies, and for Conferring LPS and Taxol Responsiveness on Mouse TLR4 by Alanine-Scanning Mutagenesis

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Identification of Mouse MD-2 Residues Important for Forming the Cell Surface TLR4-MD-2 Complex Recognized by Anti-TLR4-MD-2 Antibodies, and for Conferring LPS and Taxol Responsiveness on Mouse TLR4 by Alanine-Scanning Mutagenesis

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The expression of MD-2, which associates with Toll-like receptor (TLR) 4 on the cell surface, confers LPS and LPS-mimetic Taxol responsiveness on TLR4. Alanine-scanning mutagenesis was performed to identify the mouse MD-2 residues important for conferring LPS and Taxol responsiveness on mouse TLR4, and for forming the cell surface TLR4-MD-2 complex recognized by anti-TLR4-MD-2 Ab MTS510. Single alanine mutations were introduced into mouse MD-2 (residues 17–160), and the mutants were expressed in a human cell line expressing mouse TLR4. Mouse MD-2 mutants, in which a single alanine mutation was introduced at Cys37, Leu71, Leu78, Cys95, Tyr102, Cys105, Gln113, Val117, Pro127, Phe129, Gln136, Ile138, Leu146, Cys148, or Thr152, showed dramatically reduced ability to form the cell surface mouse TLR4-mouse MD-2 complex recognized by MTS510, and the mutants also showed reduced ability to confer LPS and Taxol responsiveness. In contrast, mouse MD-2 mutants, in which a single alanine mutation was introduced at Tyr14, Tyr46, Gln85, Val82, Ile83, Phe129, Pro127, Gly129, Ile138, Ile150, and His155 showed normal ability to form the cell surface mouse TLR4-mouse MD-2 complex recognized by MTS510, but their ability to confer LPS and Taxol responsiveness was apparently reduced. These results suggest that the ability of MD-2 to form the cell surface mouse TLR4-mouse MD-2 complex recognized by MTS510 is essential for conferring LPS and Taxol responsiveness on TLR4, but not sufficient. In addition, the required residues at codon numbers 34, 85, 101, 122, and 153 for the ability of mouse MD-2 to confer LPS responsiveness are partly different from those for Taxol responsiveness. The Journal of Immunology, 2003, 170: 413–420.
LPS. These findings, taken together, suggest that TLR4 and MD-2 constitute the central part of the LPS receptor complex.

Resolving how MD-2 contributes to formation of the LPS receptor complex is an important issue. The ability of MD-2 to form a complex with TLR4 on the cell surface is believed to be important for its ability to confer ligand responsiveness on TLR4. However, the amino acid residues of MD-2 that are important for cell surface complex formation with TLR4 and those that are important for ligand responsiveness of the TLR4-MD-2 complex have not been identified systematically. In this study, we addressed these issues by means of a strategy called alanine-scanning mutagenesis (21).

Materials and Methods

Reagents

Taxol from Taxus brevifolia was purchased from Sigma-Aldrich (St. Louis, MO). LPS prepared from Escherichia coli 0111:B4 was purchased from List Biological Laboratories (Campbell, CA). Dishes, 100 mm in diameter, 24- and 6-well, were purchased from Corning Japan (Tokyo, Japan). Oligonucleotides were prepared commercially by Qiagen (Valencia, CA). MTS510 mAbs (11) were purchased from Medical and Biological Laboratories (Nagoya, Japan). FBS was purchased from Atlanta Biologicals (Norcross, GA). DMEM and PBS (pH 7.4, without CaCl₂ and MgCl₂) were purchased from Invitrogen (San Diego, CA). Prestained molecular mass standards and polyacrylamide gel were purchased from Bio-Rad (Hercules, CA). All other chemicals used were of reagent grade or better.

Stable transfectants and cell culture

HEK 293 cells introduced with an NF-kB-dependent luciferase reporter construct (named 293/luc), and HEK 293 cells stably expressing a recombinant mouse TLR4 bearing a flag and a 6 X His tag at its C-terminal (named 293/mTLR4/luc) were generated previously (10). The HEK 293 cell lines were maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml), and streptomycin sulfate (100 µg/ml) under a 5% CO₂ atmosphere at 100% humidity and 37°C.

Expression constructs

pEFBOS (22) vector-based expression constructs, which encode a recombinant mouse TLR4 or mouse MD-2 cDNA bearing a flag tag followed by a 6 X His tag at the C-terminal (11), were provided by Dr. K. Miyake (University of Tokyo, Tokyo, Japan), and were named in this study mTLR4/h and mMD-2/h, respectively. Mouse MD-2 mutant cDNAs were generated by PCR-based overlap extension (23) with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). The sequences of the PCR primers are available upon request. Expression construct mMD-2/h was used as a PCR template, and every generated mutant MD-2 cDNA was designed to bear a flag tag followed by a 6 X His tag at its C-terminal. The generated mutant MD-2 cDNAs were cloned into the XhoI and NotI sites of pEFBOS, and each mutant MD-2 expression construct is abbreviated as the wild-type residue (single letter amino acid designation) followed by the codon number and mutant residue (typically alanine). The cDNA inserts in the expression constructs were verified by sequencing with an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA). Control expression vector pEFBOS-h was generated previously (10).

Transient transfection

Cells (3 X 10⁶/ml) were seeded into the wells of 24-well (1 ml/well) or 6-well (5 ml/well) dishes. After cultivation overnight, the cells were transfected with plasmids (0.3 µg/well of a 24-well dish, or 1.5 µg/well of a 6-well dish) using FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN). After a 24- to 48-h transfection, the culture medium was replaced with fresh medium. The plasmids used for the transfection were purified with a Wizard PureFecction Plasmid DNA Purification system (Promega, Madison, WI).

FIGURE 1. Cell surface TLR4-MD-2 complex formation on HEK 293 cells. A. 293/luc cells (9 X 10⁵ cells/30 ml of culture medium in a 100-mm diameter dish) were transfected with 8.7 µg of control expression vector pEFBOS (a), 2.9 µg of pEFBOS (b), 2.9 µg of mTLR4/h and 5.8 µg of pEFBOS (c), or 2.9 µg of mTLR4/h and 5.8 µg of mMD-2/h (d). Three days after transfection, the cell surface was stained with MTS510 mAbs followed by use of an Enzymatic Amplification Staining kit. B. 293/mTLR4/h cells cultivated in a 6-well dish were transfected with pEFBOS (a) or mMD-2/h (b). Three days after transfection, the cells were stained with MTS510 mAbs followed by fluorescein-conjugated anti-rat IgG. The average signal intensity of each event is shown in the panel (signal average). C. 293/ mTLR4/h luc cells cultivated in a 24-well dish were transfected with pEFBOS (Control vector) or mouse MD-2/h (mouse MD-2). Three days after transfection, the cells were cultivated in culture medium containing 0.5% DMSO (medium), or 0.5% DMSO and 33 ng/ml LPS or 10 µM Taxol for 6 h, and then luciferase activity was measured. The columns indicate the averages for duplicate wells.
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**FIGURE 2.** (continues)
codon numbers are 100% (wild type; wt). was replaced by alanine. Amino acid residue of mouse MD-2 at codon
values in A of that of cells transfected with mouse MD-2fh. Data shown are the aver-
B
signal intensity, as shown in Fig. 1, and was expressed as the percentage
C
complex formation was measured as MD-2 expression-induced average
of cells transfected with mouse MD-2fh. MTS510-recognized cell surface
B
C
. LPS
, or staining with MTS510 mAbs, as described in Fig. 1
Determination of important residues of mouse MD-2 for
FIGURE 2. Determination of important residues of mouse MD-2 for
conferring LPS and Taxol responsiveness on mouse TLR4, and for forming
the cell surface TLR4-MD-2 complex recognized by MTS510 by alanine-
scanning mutagenesis. A, Mouse MD-2fh (MD-2), pEFBOS
(Vector), and mutant mouse MD-2 expression constructs were transfected into 293/
mTLR4/luc cells, followed by stimulation with LPS or Taxol, as described
in Fig. 1C, or staining with MTS510 mAbs, as described in Fig. 1B. LPS
or Taxol-induced luciferase activity was expressed as the percentage of that
of cells transfected with mouse MD-2fh. MTS510-recognized cell surface
complex formation was measured as MD-2 expression-induced average
signal intensity, as shown in Fig. 1B, and was expressed as the percentage of
that of cells transfected with mouse MD-2fh. Data shown are the aver-
ages ± SD for three or more independent experiments. B, The average
values in A are plotted against codon numbers of mouse MD-2 residues that
was replaced by alanine. Amino acid residue of mouse MD-2 at codon
numbers 30, 107, 135, 137, and 139 is Ala; therefore, the values for these
codon numbers are 100% (wild type; wt).
Luciferase assay
Cells were lysed with cell culture lysis reagent (Promega), and luciferase
activity in the cell lysates was measured with a Luciferase assay system
(Promega), as described previously (14).
Cell surface staining
Unless indicated otherwise, all procedures were performed at 4°C or on ice.
Three days after transfection, the cells in a 6-well dish were washed with
2 ml of buffer A (PBS containing 3% (v/v) FBS and 0.5 mg/ml NaN3), and
then collected. The cells were incubated in buffer A containing 20 μg/ml
MTS510 mAbs for 30 min. After incubation, the cells were washed with 1
ml of buffer A twice, and then incubated with buffer A containing 30 μg/ml
dichlorotriazinyl amino fluorescein-conjugated anti-rat IgG (Immunotech,
Luminy, France) for 30 min. Then the cells were washed with 1 ml of
buffer A twice and collected. The collected cells were suspended in 500 μl
of buffer A containing propidium iodide (10 μg/ml), and then analyzed
with a FACSCalibur (BD Biosciences, Mountain View, CA). Alterna-

SDS-PAGE and Western blotting
Each cell lysate was incubated with Ni-NTA agarose (Qiagen) under de-
naturing conditions according to the manufacturer’s instructions. The pro-
teins absorbed to the resin were eluted with 0.1 M sodium-phosphate buffer
(pH 8.0) containing 8 M urea and 250 mM imidazole. The eluted proteins
were fractionated by SDS-PAGE (5–20% gradient) under reducing condi-
tions (24). For Western blot analysis, proteins separated by SDS-PAGE
were electrophoritically transferred onto nitrocellulose membranes (Schleicher & Schuell,
Keene, NH) in 25 mM Tris/192 mM glycine/0.02% SDS/20% methanol at
6.6 V/cm for 18 h. Then each blot was incubated with Tetra-His Ab
(Qiagen), and subsequently with anti-mouse IgG linked to HRP (Amer-
sham Pharmacia Biotech, Piscataway, NJ). Cross-reactive proteins were
detected with ECL Western blotting detection reagents (Amersham Phar-
macia Biotech).

Results
Screening of important amino acid residues of mouse MD-2 for
forming the cell surface TLR4-MD-2 complex recognized by anti-
TLR4-MD-2 Abs, and for conferring LPS and Taxol
responsiveness on mouse TLR4
Cell surface mouse TLR4-MD-2 complex formation has been de-
tected on a Ba/F3 stable transfectant expressing both mouse TLR4
and mouse MD-2 by flow cytometry with an anti-mouse TLR4 mAb,
MTS510, which preferentially reacts with mouse TLR4, which is as-
associated with mouse MD-2 (11). Similarly, on the HEK 293 cell
surface, MTS510 reacts with the mouse TLR4-MD-2 complex
more than mouse TLR4 alone (Fig. 1A). The average signal inten-
sity apparently increases (10.01 U) on expression of mouse MD-2
in 293/mTLR4/luc cells, a HEK 293 cell line stably expressing
mouse TLR4, indicating that the TLR4-MD-2 complex is formed on
the surface of 293/mTLR4/luc cells (Fig. 1B). To identify the
important amino acid residues of mouse MD-2 for complex for-
mation with TLR4 on the cell surface, we generated expression
constructs of mouse MD-2 mutants in which amino acid residues
from Glu17 to C-terminal Asn160 were individually replaced by
alanine. Alanine is usually chosen for replacement because it elim-
ates the side chain beyond the β carbon yet does not alter the
main-chain conformation or have an extreme electrostatic or steric
effect (21, 25). Because MD-2 is a secretory protein (26), and the
site between Thr16 and Glu 17 of mouse MD-2 was predicted to be
a possible cleavage site of the leader sequence with the PSORT II
program (http://psort.imis.u-tokyo.ac.jp/), we analyzed the amino
acid residues from Glu17 to C-terminal Asn160. The expression
constructs were introduced into 293/mTLR4/luc cells, and cell surface
TLR4-MD-2 complex formation was measured by flow cyto-
metry using MTS510 (Fig. 2). Expression of mouse MD-2 con-
fers LPS and Taxol responsiveness on 293/mTLR4/luc cells (Ref. 10
and Fig. 1C), and this responsiveness depends on the expression
of mouse TLR4 (10). The LPS and Taxol responsiveness of 293/
mTLR4/luc cells with the mutant MD-2 expression constructs in-
roduced was examined by measuring LPS or Taxol-induced NF-
κB-dependent reporter (luciferase) activation (Fig. 2). Many MD-2
mutants showed reduced ability to confer LPS and/or Taxol re-
ponsiveness on TLR4, and to form the TLR4-MD-2 complex that
was detected by MTS510, and the critical residues for the re-
ponses to LPS and/or Taxol, and for the formation of the TLR4-
MD-2 complex recognized by MTS510 were not clustered in a
particular region of mouse MD-2 (Fig. 2B).
The ability of MD-2 to form a complex with TLR4 on the cell surface is essential for its ability to confer LPS and Taxol responsiveness on TLR4.

The reactivity of MTS510 mAbs, which specifically react with the cell surface TLR4-MD-2 complex, was plotted against LPS-induced luciferase activity (A) or Taxol-induced luciferase activity (B). The values for complex formation and luciferase activity are the average values shown in Fig. 2. C and D, Magnifications of the regions demarcated by dotted lines in A and B, respectively. E, 293/mTLR4/luc cells cultivated in 6-well dishes were transfected with pEFBOS (Vector), mouse MD-2h (mouse MD-2), or mutant mouse MD-2 expression constructs. The mutant mouse MD-2 expression constructs and mouse MD-2h were designed to express recombinant MD-2 proteins that bear a 6× His tag at their C-terminal, as described under Materials and Methods. The expressed recombinant protein in each cell lysate was absorbed to Ni-NTA agarose and then subjected to Western blotting. Proteins reactive to anti-His tag Abs were detected. The sizes of molecular mass standards are indicated on the left. Because MD-2 was glycosylated (32), several signals appeared around 30 kDa, as described previously (11).

Cell surface complex formation with TLR4 is not sufficient for MD-2 to confer LPS or Taxol responsiveness on TLR4.

The reactivity of MTS510 mAbs with 293/mTLR4/luc cells expressing the Y34A, Y36A, G59A, V82A, I117A, P118A, F119A, E136A, I138A, L146A, C148A, and T152A mutants was <55% of that of cells expressing mouse MD-2 (Figs. 2 and 3, A and B). Although the abilities of these mutants to form a complex with TLR4 on the cell surface were similar to or somewhat lower than that of mouse MD-2, their abilities to confer LPS and Taxol responsiveness on 293/mTLR4/luc cells were apparently lower than that of mouse MD-2 (Fig. 3, A and B). These results suggest that cell surface complex formation with TLR4 is not sufficient for MD-2 to confer LPS or Taxol responsiveness on TLR4.

Furthermore, we examined the amino acid residues replaced by alanine with which the ability to form a complex with mouse TLR4 was retained, but with which the ability to confer both LPS and Taxol responsiveness on mouse TLR4 was apparently reduced. Ile85, Ile153, and Tyr159 of mouse MD-2 were individually replaced by Met, Leu, and Trp, respectively, and then the ability to confer LPS and Taxol responsiveness on 293/mTLR4/luc cells was...
Ile\textsuperscript{153} and Tyr\textsuperscript{34} by Ala reduces the ability of mouse MD-2 to confer both LPS and Taxol responsiveness on mouse TLR4. These results, taken together, suggest that the amino acid residues at codon numbers 34, 85, and 153 of mouse MD-2 are important for conferring both LPS and Taxol responsiveness on mouse TLR4, and that the required amino acid residues at these positions for conferring LPS responsiveness are partly different from those for Taxol responsiveness.

A single alanine mutation at Asp\textsuperscript{101} or Glu\textsuperscript{122} of mouse MD-2 reduces its ability to confer Taxol responsiveness on mouse TLR4, but does not affect its ability to confer LPS responsiveness.

Previously, we demonstrated that a single mutation at Gln\textsuperscript{22} of mouse MD-2 reduces its ability to confer Taxol responsiveness on mouse TLR4, but does not affect its ability to confer LPS responsiveness (10). In addition to a single alanine mutation at Gln\textsuperscript{22}, single alanine mutations at Asp\textsuperscript{101} and Glu\textsuperscript{122} also reduced the ability of mouse MD-2 to confer Taxol responsiveness, but not LPS responsiveness (Fig. 5A). Moreover, we examined its ability to confer LPS and Taxol responsiveness on TLR4 by measuring dose responses against LPS or Taxol stimulation. Consistent with the previous findings, 293/mTLR4/luc cells expressing the Q22A mutant showed lower Taxol sensitivity than that of cells expressing mouse MD-2, and cells expressing the Q22A mutant showed similar LPS sensitivity to that of cells expressing mouse MD-2 (Fig. 5B). Cells expressing the D101A or E122A mutant showed apparently lower Taxol sensitivity than ones expressing mouse MD-2, and their LPS sensitivity was similar to that of cells expressing mouse MD-2 (Fig. 5, C and D). In contrast, cells expressing the R157A mutant showed lower LPS sensitivity to LPS than ones expressing mouse MD-2, and they showed similar Taxol sensitivity to that of cells expressing mouse MD-2 (Fig. 5, A and E).

**Discussion**

By means of alanine-scanning mutagenesis of mouse MD-2, here we have found important residues for conferring LPS and Taxol responsiveness on mouse TLR4, and for formation of the cell surface TLR4-MD-2 complex that is recognized by MTS510, which specifically reacts with the cell surface mouse TLR4-MD-2 complex. In addition, we have shown that the required amino acid residues at codon numbers 34, 85, and 153 for the ability of mouse MD-2 to confer LPS responsiveness on mouse TLR4 are partly different from those for Taxol responsiveness, and that a single alanine mutation at Asp\textsuperscript{101} or Glu\textsuperscript{122} reduces the ability of mouse MD-2 to confer Taxol responsiveness on mouse TLR4, but does not affect its ability to confer LPS responsiveness. A structural difference between the LPS lipid A moiety (2) and Taxol (27) may account for the difference in the MD-2 residues required for conferring their responsiveness on TLR4. Previously, we showed that MD-2 is responsible for the species-specific action of Taxol, and that Gln\textsuperscript{22} of mouse MD-2, which is not conserved between man and mouse, is important for this action of Taxol (10, 14). Glu\textsuperscript{122} of mouse MD-2 is not conserved in man and mouse, but Asp\textsuperscript{101} is. Because Taxol is derived from a plant (27), it might not be a natural ligand for the TLR4-MD2 complex. The conserved Asp\textsuperscript{101} might be important for other TLR4 ligands, such as lipoteichoic acid (28), flavolipin (29), and heat shock protein 60 (30).

We have shown that alanine substitution at Tyr\textsuperscript{34}, Tyr\textsuperscript{36}, Gly\textsuperscript{59}, Val\textsuperscript{82}, Ile\textsuperscript{85}, Phe\textsuperscript{126}, Pro\textsuperscript{127}, Gly\textsuperscript{129}, Ile\textsuperscript{153}, Ile\textsuperscript{154}, or His\textsuperscript{155} of mouse MD-2 apparently reduces its ability to confer LPS and Taxol responsiveness on TLR4, but does not affect its ability to form a complex with TLR4 so much. These amino acid alterations may affect the direct interaction between MD-2 and ligands, or
may induce conformational changes of TLR4-MD-2 complex that are important for the direct interaction with ligands or for inducing intracellular signaling. The precise molecular mechanisms underlying the involvement of these amino acid residues conferring ligand responsiveness on TLR4 remain to be for further analysis.

In this study, we systemically analyzed mouse MD-2 residues that affect the formation of the TLR4-MD-2 complex using MTS510 Abs, which react more strongly with the mouse TLR4-MD-2 complex than TLR4 alone. We found that some mouse MD-2 mutants showed dramatically reduced ability to form the cell surface mouse TLR4-mouse MD-2 complex recognized by MTS510. The Ab was raised against mouse TLR4, was reported to react with TLR4 but not with MD-2, and was suggested to recognize the specific conformation of mouse TLR4 that is associated with mouse MD-2 (11). Therefore, 293/mTLR4/luc cells that express a mutant mouse MD2 but do not react with MTS510 might be defective in the TLR4-MD-2 complex formation on the cell surface because of a defect in the physical association of the mutant MD-2 with TLR4, and/or a defect in the translocation of the TLR4-MD-2 complex, which has been shown to be formed in the endoplasmic reticulum (26) to the cell surface. Alternatively, such cells might have lost the conformational epitope recognized by the Ab. In addition, we found that the mouse MD-2 mutants showed reduced ability to confer LPS and Taxol responsiveness. All things considered, we suggest that the ability of MD-2 to form the cell surface TLR4-MD-2 complex is essential for its ability to confer LPS and Taxol responsiveness on TLR4, but not sufficient.

Cysteine residues, which form intra and intermolecular disulfide bonds, are generally thought to be important for the molecular structure. Human MD-2 contains seven cysteine residues, which are conserved between mouse and man, and these residues are important for the formation of large disulfide-linked oligomers (26, 31). Our results showed that Cys residues, such as Cys137, Cys95, Cys105, and Cys148 of mouse MD-2 are important for conferring LPS and Taxol responsiveness on mouse TLR4 and for forming the cell surface TLR4-MD-2 complex recognized by MTS510. In contrast, substitution of Cys133 by Ala did not affect the ability to confer LPS and Taxol responsiveness on TLR4, or the ability to form the TLR4-MD-2 complex recognized by MTS510 so much.

Structural analysis of MD-2 and the TLR4-MD-2 complex is essential for further understanding of the molecular mechanisms underlying the recognition of ligands and signal transduction. We believe that our functional analysis of MD-2 residues should be helpful for the modeling of the structure of MD-2.
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References