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N-Linked Glycosylations at Asn^{26} and Asn^{114} of Human MD-2 Are Required for Toll-Like Receptor 4-Mediated Activation of NF-κB by Lipopolysaccharide

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MD-2 is physically associated with Toll-like receptor 4 (TLR4) and is required for TLR4-mediated LPS signaling. Western blotting analysis revealed the presence of three forms of human (h)MD-2 with different electrophoretic mobilities. After *N*-glycosidase treatment of the cellular extract prepared from cells expressing hMD-2, only a single form with the fastest mobility was detected. Mutation of either one of two potential glycosylation sites (Asn^{26} and Asn^{114}) of MD-2 resulted in the disappearance of the slowest mobility form, and only the fastest form was detected in hMD-2 carrying mutations at both Asn^{26} and Asn^{114}. Although these mutants were expressed on the cell surface and maintained its ability to associate with human TLR4, these mutations or tunicamycin treatment substantially impaired the ability of MD-2 to complement TLR4-mediated activation of NF-κB by LPS. LPS binding to cells expressing CD14, TLR4, and MD-2 was unaffected by these mutations. These observations demonstrate that hMD-2 undergoes *N*-linked glycosylation at Asn^{26} and Asn^{114}, and that these glycosylations are crucial for TLR4-mediated signal transduction of LPS. *The Journal of Immunology*, **2001, 167: 3354–3359.**

Lipopolysaccharide is a component of the outer membrane of Gram-negative bacteria and activates a variety of cells, including monocytes and macrophages (1, 2). This activation induces the production of many cytokines, such as IL-1, IL-8, and TNF-α, and plays a major role in septic shock in humans (1). CD14 is believed to be a recognition molecule of LPS and is thought to play a central role in the activation of cells with the help of LPS-binding protein (LBP)[2] (3, 4). However, because CD14, a GPI-anchored protein, lacks a transmembrane region (5), a search was made for transmembrane molecules that transmit LPS signaling into the cytoplasm, and Toll-like receptors (TLRs) were identified as candidates (6–9).

TLRs are mammalian homologues of the *Drosophila* Toll protein, which plays critical roles in the establishment of dorsoventral polarity and the antifungal response in adult flies (10). In mammals, the TLR family consists of at least eight members. Two of them, TLR2 and TLR4, have been found to confer responsiveness to bacterial products (6, 7, 9, 11). TLR2 responds to peptidoglycans and lipoteichoic acids (12), whereas TLR4 is involved in LPS signaling. A recent study demonstrated that TLR4-mediated activation of NF-κB by LPS required MD-2, a novel accessory molecule expressed on the cell surface and associated with TLR4 (13).

Human (h)MD-2 consists of 160 amino acids residues with a predicted molecular mass of 18 kDa, and there are two potential *N*-linked glycosylation sites in this amino acid sequence. Although the general role of glycosylation of protein molecules has not been completely established, many functions of particular glycosylations have been reported. These functions include modulation of enzyme and hormone activity, regulation of intracellular traffic, control of protein folding, ligand recognition, and cell-cell interaction (see Ref. 14 for review). However, the structural and functional implications of glycosylation have not been elucidated in regard to the hMD-2 molecule. In this study, we found that hMD-2 undergoes *N*-linked glycosylation, and that the glycosylation is essential for hTLR4-mediated activation of NF-κB by LPS.

Materials and Methods

Cells and reagents

The human embryonic kidney 293 cell line (obtained from the Human Science Research Resource Bank, Tokyo, Japan) was grown in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (v/v) heat-inactivated FCS (Life Technologies), penicillin (100 U/ml), and streptomycin (100 μg/ml). The human monocytic cell line THP-1 was grown in the same way as the 293 cells except that RPMI 1640 (Life Technologies) was used instead of DMEM. LPS was prepared from *Escherichia coli* 03K2a2B:H2 (S type) and R3 F653 (R type) as described by Westphal et al. (15) and Galanos et al. (16), respectively, and was purified according to the methods of Hirschfeld et al. (17). An antiserum (1060) against the equine infectious anemia virus (EIAV)-tag epitope (amino acid sequence ADRRIPGTAEE) was a kind gift of Dr. N. Rice (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). A goat anti-human TLR4 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sulfosuccinimidyl-6-(bioitinamido)-6-hexanamido hexanoate (sulfo-NHS-LC-LC-biotin) and immobilized streptavidin agarose were from Pierce (Rockford, IL). PNGase F was purchased from New England Biolabs (Beverly, MA). Alexa fluor 594-conjugated LPS was purchased from Molecular Probes (Eugene, OR). Unless otherwise noted, all other chemicals were obtained from Wako Pure Chemical (Osaka, Japan).

Plasmid construction and site-directed mutagenesis

Plasmid containing human CD14 cDNA was provided by Dr. S. Yamamoto (Medical College of Oita, Oita, Japan). The coding regions of hMD-2 and hTLR4 were amplified by RT-PCR from total RNA prepared from THP-1 and human spleen total RNA (OriGene Technologies, Rockville, MD), respectively. The coding regions of hMD-2, CD14, and TLR4

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[2] Abbreviations used in this paper: LBP, LPS-binding protein; TLR, Toll-like receptor; EIAV, equine infectious anemia virus; ELAM, endothelial cell-leukocyte adhesion molecule; h, human; sulfo-NHS-LC-LC-biotin, sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate; RL-TK, *Renilla* luciferase-thymidine kinase.

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minus their predicted signal peptide sequences were subcloned downstream of a mammalian expression vector in which the preprotrypsine signal peptide sequence precedes the NH_2-terminal EIAV tag (these constructs will be referred to as pEIAV-hMD-2, pEIAV-hCD14, and pEIAV-hTLR4, respectively). Epitope tagging had no influence on the activity of hMD-2, CD14, and TLR4 in tissue culture. Luciferase reporter plasmid endothelial cell-leukocyte adhesion molecule (pELAM)-L, was constructed by inserting the PCR fragment (−730 to +52) of the E-selectin promoter into the *Sac*I-*Hin*dIII site of pGL3 Basic vector (Promega, Madison, WI). Point mutations at the potential glycosylation sites (Asn^{26} and Asn^{114}) of hMD-2 were created by PCR. One of either asparagines (N26Q or N114Q) or both (N26:114Q) was replaced with glutamine. The sequence of primers used for PCR were as follows: 5′-GGG TCT GCC AGT CAT CCG ATG CA-3′ and 5′-AAT ACT GCT TCT GAG CTT CAG TAA ATA TGG-3′ for N26Q and 5′-GAG ACT GTG CAG ACA ACA ATA TCA TTC TCC-3′ and 5′-TCC CTT CAG AGC TCT GCA AAA AGA GT-3′ for N114Q. All mutations were confirmed by automated DNA sequencing using a dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) and an ABI Prism 310 genetic analyzer (PerkinElmer Applied Biosystems, Foster City, CA).

Glycosidase treatment of cellular extracts

After plating 293 cells in 6-cm dishes, the cells were transfected by the calcium phosphate precipitation method (18) with a hMD-2 expression plasmid (5 μg), and 24 h later, the cells were lysed with a lysis buffer (10 mM HEPES-KOH, 5 mM EDTA, 0.5% Nonidet P-40, and 10 mM KCl (pH 7.9)) containing a protease inhibitor mix (Boehringer Mannheim, Mannheim, Germany) on ice for 10 min. Following centrifugation at 1000 × *g* for 5 min, the supernatant obtained was used as the cellular extract and boiled for 10 min in 0.5% SDS and 1% 2-ME. After adding 1/10 vol of 0.5 M sodium phosphate (pH 7.5) and 10% Nonidet P-40, the boiled extract was incubated with PNGase F (final concentration of 10 U/ml) at 37°C for 1 h, and then subjected to SDS-PAGE. The hMD-2 molecule was detected by Western blot analysis. The signals were visualized with an ECL system (Amersham, Arlington Heights, IL).

Biotinylation of cell surface protein

After plating 293 cells in 6-cm dishes, they were transfected by the calcium phosphate precipitation method with hMD-2 expression plasmids (5 μg each), and 24 h later, the cells were washed with PBS twice and treated with 2 ml ice-cold 0.5 mg/ml sulfo-NHS-LC-LC-biotin, a membrane-impermeable biotinylation reagent (19, 20), in PBS at 4°C for 30 min. Then, after stopping the biotinylation reaction by incubating with 20 mM glycine in PBS at 4°C for 15 min, cellular extracts were prepared with 200 μl lysis buffer as described above. The cellular extracts were diluted to 500 μl with PBS containing 0.5% Nonidet P-40 and incubated with immobilized streptavidin agarose at 4°C for 1 h. After washing with PBS containing 0.5% Nonidet P-40 three times, the agarose was boiled in SDS-PAGE sample buffer. The supernatant obtained was subjected to SDS-PAGE and Western blot analysis, and hMD-2 was detected with a rabbit anti-EIAV antiserum as described above.

Immunoprecipitation of TLR4

After seeding 293 cells in 6-cm dishes, they were transfected by the calcium phosphate precipitation method with hMD-2 and TLR4 expression plasmids (5 μg each), and 24 h later, cellular extracts were prepared as described above. The cellular extracts were diluted to 500 μl with PBS containing 0.5% Nonidet P-40, and after adding an anti-human TLR4 Ab and protein A/G-Sepharose (Pierce), the diluted cellular extracts were incubated for 1 h at 4°C with rocking. After washing with PBS containing 0.5% Nonidet P-40, the Sepharose beads were boiled in SDS-PAGE sample buffer, and the supernatant was subjected to SDS-PAGE and Western blot analysis.

Purification of rhLBP

The coding region of hLBP minus its signal sequence was amplified by RT-PCR from total RNA prepared from THP-1 and subcloned into a yeast expression vector pGAPZα (Invitrogen, Carlsbad, CA) with an N-terminal ×6 histidine tag sequence. LBP was expressed in a Pichia expression system according to the recommendation of the manufacturer (Invitrogen) and was purified on a Ni^{2+} column (Novagen, Madison, WI).

NF-κB reporter assay

After plating 293 cells in 6-well dishes (4×10^5/well), they were transfected by the calcium phosphate precipitation method with the expression plasmids indicated (0.02 μg), 0.2 μg of pELAM-L luciferase reporter plasmid, and 0.05 μg of pRL-TK (*Renilla* luciferase-thymidine kinase) (Promega) for normalization. After 24 h, cells were stimulated for 6 h in serum-free DMEM containing 100 ng/ml of human LBP, and the reporter gene activity was measured according to the recommendation of the manufacturer (Promega). Results were normalized by thymidine kinase reporter activity.

Tunicamycin treatment

After plating 293 cells (4×10^5/well) in 6-well dishes, they were transfected with hTLR4 and hMD-2 expression plasmid (2 ng) together with luciferase plasmid as described above. At 8 h after transfection, the medium was replaced with normal culture medium containing the indicated concentration of tunicamycin (WAKO Pure Chemical), and the cells were incubated at 37°C for 16 h. The cells were then stimulated with LPS for 6 h in DMEM containing the concentration of tunicamycin indicated and 10% FCS, and the relative luciferase activity was measured as described above. Results were normalized by thymidine kinase reporter activity.

LPS-binding assay

After plating 293 cells onto a coverslip precoated with rat tail collagen type I (BD Biosciences, San Jose, CA) and transfection with the plasmid indicated (5 μg), the cells were incubated with 100 ng/ml of Alexa fluor 594-conjugated LPS in DMEM containing 10% FCS at 37°C for 1 h. The cells were then washed with PBS three times and fixed with 3% paraformaldehyde in PBS (pH 7.6) for 20 min. After washing with PBS, the coverslip was mounted on a slide glass with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA) and viewed under a fluorescence microscope.

Results

The hMD-2 molecule undergoes glycosylation at Asn^{26} and Asn^{114}

An expression vector containing hMD-2 cDNA was transiently transfected in human kidney 293 cells, and cellular extracts were prepared from the cells (Fig. 1). Western blotting analysis of hMD-2 detected three forms of proteins with different electrophoretic mobility (Fig. 1, *left four lanes*). Analysis of the cellular extract prepared from the cells transfected with the control vector gave no protein signals around that molecular mass region, indicating that these signals originated from hMD-2. The apparent molecular mass of these protein signals estimated by a prestained molecular mass marker were found to be ~14, 18, and 23 kDa. To investigate the involvement of glycosylation in the appearance of the multiple forms, we treated cellular extracts prepared from 293 cells transiently expressing hMD-2 with an *N*-glycosidase, PNGase. The PNGase-treated cellular extract was analyzed for hMD-2 protein by Western blotting (Fig. 1, *left*). Only the fastest

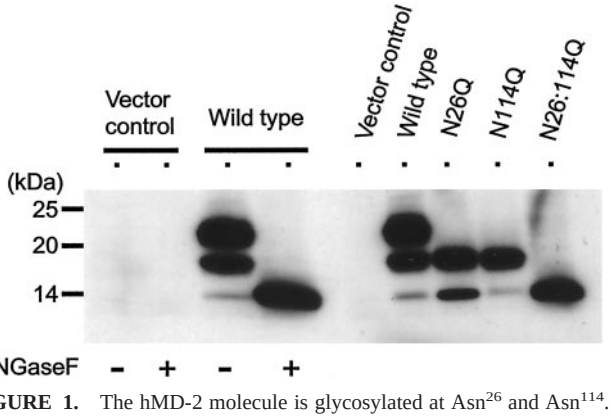

FIGURE 1. The hMD-2 molecule is glycosylated at Asn^{26} and Asn^{114}. After plating 293 cells in 6-cm dishes, they were transfected with a control vector or expression plasmids for wild-type hMD-2, N26Q, N114Q, or N26:114Q. After 24 h, cell extracts were prepared from the cells and treated with (+) or without (−) PNGase F. The extracts were then analyzed for MD-2 protein by Western blotting.

mobility form of hMD-2 was detected in the PNGase-treated extract, although three forms of hMD-2 were detected in the cellular extract treated in parallel without PNGase. Phosphatase treatment did not affect the mobility pattern (our unpublished observation). These findings suggest that glycosylation is responsible for the appearance of these forms. To confirm this, we generated hMD-2 mutant plasmids lacking one or both potential *N*-linked glycosylation sites, and these mutants were expressed in 293 cells (Fig. 1, *right five lanes*). The single mutation at Asn^{26} or Asn^{114} (named N26Q or N114Q) resulted in the disappearance of the slowest mobility form. Double mutation of both Asn^{26} and Asn^{114} (named N26:114Q) resulted in the disappearance of both the slowest and the intermediate mobility. Taken together, these results indicate that the *N*-linked glycosylation either at Asn^{26} or Asn^{114} is responsible for the appearance of the intermediate mobility form, and that the slowest mobility form is resulted from the glycosylation at both Asn^{26} and Asn^{114}.

N-*linked glycosylation of hMD-2 is crucial for LPS signaling via TLR4*

The effect of *N*-linked glycosylation of hMD-2 on LPS-induced activation of NF-κB via human TLR4 was investigated by an NF-κB-dependent luciferase reporter assay. Although 293 cells expressing only TLR4 and CD14 hardly responded to LPS, coexpression of wild-type hMD-2 resulted in strong induction of reporter activity in response to LPS. Expression of the N26Q or N114Q mutant instead of wild-type hMD-2 resulted in partial induction, and only slight induction was observed in cells expressing the double mutant N26:114Q (Fig. 2). To confirm that the lack of glycosylation of hMD-2 was responsible for the impaired activity, we next examined the effect of tunicamycin, which prevents the addition of *N*-linked glycan to polypeptide (21). The 293 cells were transiently expressed with TLR4 and hMD-2 and treated with varying concentrations of tunicamycin followed by LPS or TNF-α. In this experiment, we did not express CD14 because tunicamycin prevents the glycosylation of CD14, which may release GPI-anchored CD14 from the cell membrane and cause reduction of LPS response. Instead, we used 10% FCS as a source of soluble CD14 and LBP. Tunicamycin inhibited the LPS-induced activation of NF-κB in a concentration-dependent manner, whereas the TNF-α-induced activation was unaffected by the tunicamycin treatment

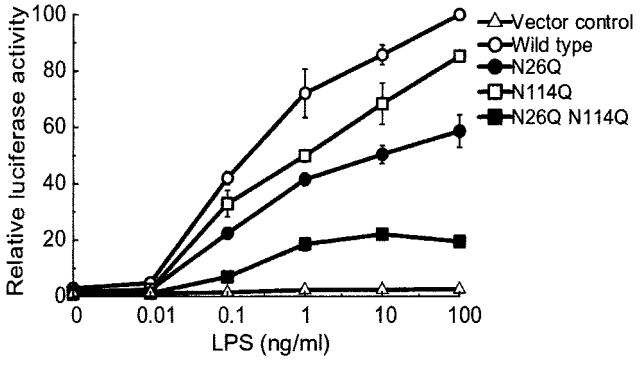

FIGURE 2. Lack of glycosylation impaired the ability of MD-2 to complement TLR4-mediated activation of NF-κB by LPS. The 293 cells were transfected with a hTLR4, a hCD14 plasmid, and either a vector control (△), wild-type hMD-2 (○), N26Q (●), N114Q (□), or N26:114Q (■) plasmids, together with pELAM-L and pRL-TK. After 24 h, cells were stimulated with LPS in serum-free DMEM containing human LBP (100 ng/ml) for 6 h, and luciferase activity was measured. Relative luciferase activity was normalized by thymidine kinase promoter activity. Data are presented as the means ± SE from at least three independent experiments.

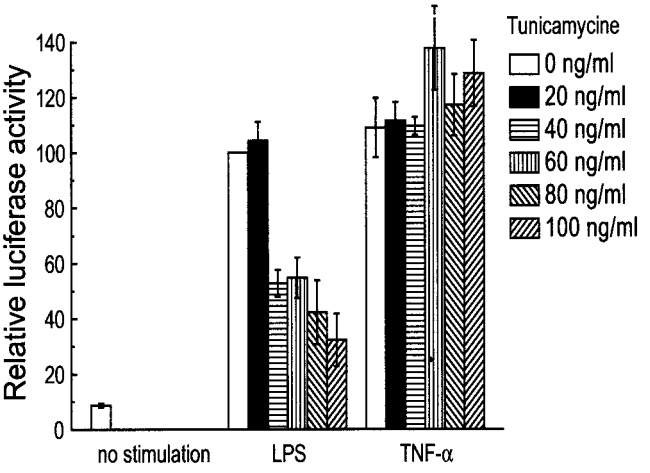

FIGURE 3. Tunicamycin treatment inhibited LPS-induced activation of NF-κB in cells expressing MD-2 and TLR4. The 293 cells were transfected with wild-type hMD-2 and hTLR4 plasmids, together with pELAM-L and pRL-TK, and the cells were treated with tunicamycin as described in *Materials and Methods*. Following treatment with the concentration of tunicamycin indicated, cells were stimulated with LPS (10 ng/ml) or TNF-α (10 ng/ml) in DMEM containing 10% FCS for 6 h, and luciferase activity was measured. Relative luciferase activity was normalized by the thymidine kinase promoter activity. Data are presented as the means ± SE from at least three independent experiments.

(Fig. 3). Tunicamycin was cytotoxic at concentrations above 100 ng/ml. These results indicate the important role of *N*-linked glycosylation of hMD-2 for LPS-induced activation of NF-κB via TLR4.

MD-2 mutants lacking glycosylation site(s) are still expressed on the cell surface

Glycosylation is known to often play a crucial role in the folding and trafficking of membrane glycoproteins (22–25). Therefore, to determine the influence of *N*-linked glycosylation on cell surface expression of hMD-2, we expressed the wild-type and mutant hMD-2 proteins into 293 cells and labeled cell surface proteins with a membrane-impermeable biotin. The biotinylated proteins were collected with streptavidin-agarose, electrophoresed by SDS-PAGE, and finally, hMD-2 was detected by Western blotting. A comparable amount of hMD-2 proteins was detected in the streptavidin-precipitates obtained from cells expressing either wild-type or mutant MD-2 proteins, whereas MD-2 was not detected without biotinylation (Fig. 4). This result demonstrates that hMD-2 is still expressed on the cell surface without glycosylation at Asn^{26} and Asn^{114}.

MD-2 mutants lacking glycosylation site(s) still associate with TLR4

To determine whether *N*-linked glycosylation participates in the association of hMD-2 with TLR4, immunoprecipitation was conducted by using an anti-human TLR4 Ab. The wild-type or mutant (N26Q, N114Q, and N26:114Q) MD-2 proteins were expressed in 293 cells with or without expression of TLR4 (Fig. 5, *lower lane*). TLR4 was immunoprecipitated with its Ab, and coprecipitated hMD-2 was detected by Western blotting. The hMD-2 molecule was not coprecipitated when TLR4 alone or hMD-2 alone was expressed (Fig. 5, *upper panel*, *lanes 1* and *2*). When both TLR4 and MD-2 were expressed, three forms of hMD-2 proteins were

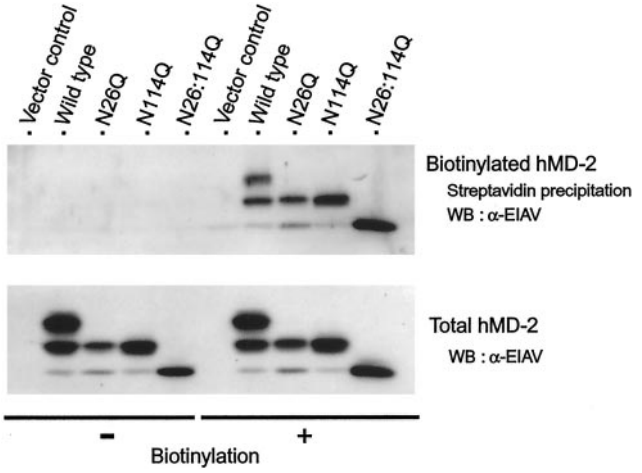

FIGURE 4. Cell surface expression of hMD-2 mutants. The 293 cells were transfected with either a control vector or the MD-2 plasmid indicated. After 24 h, cell surface proteins were biotinylated with a membrane-impermeable biotinylating reagent, sulfo-NHS-LC-LC-biotin. Cell extracts were prepared from the cells and divided into two portions. Biotinylated proteins were collected from one portion of the extracts with immobilized streptavidin agarose. The agarose beads were boiled in 2× SDS-PAGE sample buffer for 5 min, and the supernatants (*upper panel*) and cell extracts set aside (*lower panel*) were analyzed for MD-2 by Western blotting. WB, Western blot.

coprecipitated. The hMD-2 mutants that lack one or both *N*-glycosylation site(s) were also coprecipitated with TLR4. These results indicate that hMD-2 that has not been glycosylated at Asn^{26} and Asn^{114} is still capable of associating with TLR4.

N-*linked glycosylation of hMD-2 is not involved in LPS binding*

We next examined LPS binding by using Alexa fluor 594-labeled LPS (Fig. 6), because lack of glycosylation of the hMD-2 molecule may affect LPS-binding ability. The binding study was conducted in the presence of 10% FCS to prevent nonspecific binding of labeled LPS. No detectable bindings were observed in 293 cells transfected with either a vector control, TLR4, wild-type hMD-2, N26:114Q, wild-type hMD-2 plus TLR4, or N26:114Q plus TLR4.

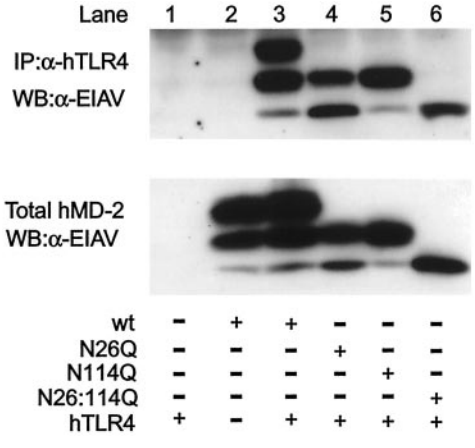

FIGURE 5. The hMD-2 mutants lacking glycosylation sites still associates with TLR4. The 293 cells were transfected with the plasmids indicated (shown at the *bottom of the panels*). After 24 h, cell extracts were prepared from the cells, and a portion of the extracts was subjected to immunoprecipitation with anti-hTLR4 IgG. Both the precipitates (*upper panel*) and the cell extracts set aside (*lower panel*) were analyzed for MD-2 by Western blotting. IP, Immunoprecipitation; WB, Western blot; wt, wild type.

However, the bindings were clearly detectable in 293 cells expressing either wild-type hMD-2 plus CD14 or wild-type hMD-2 plus TLR4 plus CD14. These bindings were still detectable when N26:114Q was expressed instead of wild-type hMD-2, indicating no significant role of glycosylation of hMD-2 in the LPS binding. Taken together, these findings indicate that *N*-linked glycosylations at Asn^{26} and Asn^{114} in hMD-2 are crucial for TLR4-mediated signal transduction of LPS.

Discussion

MD-2 was identified as a cell surface molecule that associates with TLR4 and confers LPS responsiveness on TLR4 (13). The hMD-2 molecule, including its signal peptide sequence, consists of 160 amino acids with a predicted molecular mass of 18 kDa. Western blot analysis of hMD-2 expressed in 293 cells revealed the presence of three forms of hMD-2 with apparent molecular masses of ~14, 18, and 23 kDa, respectively. Shimazu et al. (13) reported that hMD-2 is detectable as multiple bands on Western blot analysis. The PROSIT scan analysis revealed that there are two potential *N*-linked glycosylation sites and several phosphorylation sites in the amino acid sequence of hMD-2. *N*-glycosidase treatment, but not phosphatase treatment, reduced the molecular mass of the two slower mobility forms, resulting in the detection of only the fastest mobility form. In addition, our mutational analysis at the potential glycosylation sites clearly demonstrated that the glycosylation at Asn^{26} and Asn^{114} caused the molecular mass shift.

N-linked glycosylation is a cotranslational modification found in most cell surface proteins, but the precise function of the carbohydrate on these proteins is not well understood (26). Until now, at least two categories of functions of glycosylation were known. One consists of physicochemical functions, which include modifications of solubility, electrical charge, and mass, and control of protein folding and stabilization of protein conformation (14), all of which are important for trafficking and correct folding of membrane glycoproteins. The other consists of biological functions. Recent reports have provided evidence that *N*-linked glycosylation may be required for ligand recognition or signaling (27–30). However, what role *N*-linked glycosylation plays in ligand binding and signaling remains uncertain. *N*-linked glycosylation may stabilize a conformation required for binding, or oligosaccharides themselves may be an essential part of the binding site. To explore the function of glycosylation of hMD-2, we generated hMD-2 mutants lacking one or both potential *N*-linked glycosylation sites. The ability of hMD-2 to complement LPS-induced NF-$\kappa\beta$ activation via TLR4 was substantially impaired when the hMD-2 mutant lacking both *N*-linked glycosylation sites was expressed instead of wild-type hMD-2. The possibility that lack of glycosylation of hMD-2 impaired its membrane expression can be excluded, because a comparable level of the membrane surface expression was observed between the wild-type and mutant hMD-2 proteins (Fig. 4). It is unlikely that the mutations at Asn^{26} and/or Asn^{114} of hMD-2 disrupted its secondary structure, leading to loss of function of hMD-2, because hMD-2 carrying these mutations still retained the ability to associate with TLR4 (Fig. 5). In addition, tunicamycin treatment, which prevents *N*-linked glycosylation, greatly inhibited the LPS-induced NF-κB activation in cells expressing TLR4 and MD-2. Thus, these results indicate that the glycosylation of hMD-2 plays a crucial role in the LPS response via TLR4. TLR4 also possesses potential glycosylation sites. Overexpression of TLR4 is known to increase constitutive NF-κB activity (31). We observed that tunicamycin treatment did not inhibit the constitutive activation of NF-κB induced by overexpression of TLR4 (data not shown). This indicates that the lack of glycosylation of TLR4 does not affect at least one of the functions

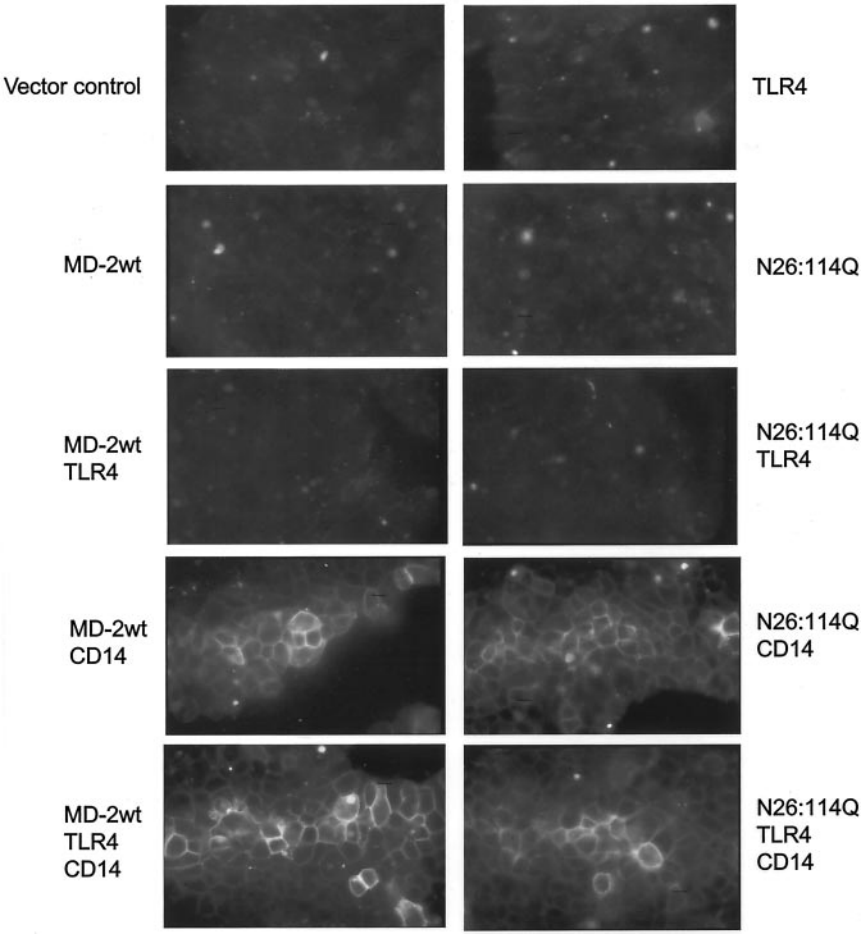

FIGURE 6. Lack of glycosylation of hMD-2 does not affect LPS binding. The 293 cells were transfected with a control vector or the expression plasmids indicated, and LPS-binding was detected with Alexa fluor 594-conjugated LPS as described in *Materials and Methods*.

of TLR4, although this does not exclude the possibility that the lack of glycosylation of TLR4 may affect LPS-induced activation of TLR4. In contrast, TNF-α-induced activation of NF-κB was unaffected by tunicamycin treatment (Fig. 3), even though the p60 TNFR, which is reported to be involved in the activation of NF-κB (32), possesses three potential *N*-linked glycosylation sites (33). This may reflect a selective role of glycosylation of MD-2 in the LPS response.

Shimazu et al. (13) failed to detect cell surface expression of hMD-2 when it was stably expressed in Ba/F3 cells without expression of TLR4, suggesting that MD-2 is anchored in the membrane via physical association with TLR4. However, in our study, we detected cell surface expression of hMD-2 without TLR4 in 293 cells. It is unlikely that the biotinylation reagent we used labeled intercellularly located MD-2 because of the following reasons: 1) This reagent is well characterized (19, 20) not to pass through cell membrane due to its strong negative charge (SO_3^-); 2) we performed the biotinylation at 4°C to prevent endocytosis, and we stopped the biotinylation reaction by adding glycine before lysing the cells; and 3) we were unable to detect the biotinylation of a cytosolic protein, IκB-α, with this biotinylation reagent. Recently, da Silva Correia et al. (34) also observed that MD-2 was clearly detectable on the cell surface without TLR4 expression using FACS analysis when MD-2 was transiently expressed in 293 cells. Therefore, the difference between our result and the result of Shimazu et al. (13) seems to be attributable to the difference in the level of MD-2 expression between stable and transient transfection. Higher expression can be obtained with a transient transfection system, and that may have led to the detection of MD-2 expressed on the cell surface without anchoring to TLR4, although coexpression of TLR4 may increase cell surface expression of MD-2.

It is unknown how the glycosylation of MD-2 participates in the LPS signaling via TLR4. At least the mutations of potential glycosylation sites of MD-2 did not affect the ability of MD-2 to associate with TLR4 or LPS binding to cells expressing TLR4, CD14, and MD-2 (Figs. 5 and 6). LPS binding was inhibited by a 100-fold excess of unlabeled LPS (data not shown), and no binding was detected in 293 cells transfected with vector alone (Fig. 6). These results indicate that the binding we detected reflects the specific binding of LPS. Akashi et al. (35) reported that a conformational change may occur in mouse TLR4 as a result of the association of MD-2. The glycosylation of hMD-2 may participate in stabilizing a certain conformation of TLR4 that allows TLR4 to transmit LPS signaling. This remains to be clarified.

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