The lipopolysaccharide (LPS) receptor is a multi-protein complex that consists of at least three proteins, CD14, TLR4, and MD-2. Because each of these proteins is glycosylated, we have examined the functional role of N-linked carbohydrates of both MD-2 and TLR4. We demonstrate that MD-2 contains 2 N-glycosylated sites at positions Asn26 and Asn114, whereas the amino-terminal ectodomain of human TLR4 contains 9 N-linked glycosylation sites. Site-directed mutagenesis studies showed that cell surface expression of MD-2 did not depend on the presence of either N-linked site, whereas in contrast, TLR4 mutants carrying substitutions in Asn26 or Asn575 failed to be transported to the cell surface. In contrast, TLR4 mutants carrying substitutions in Asn26 or Asn575 failed to be transported to the cell surface. Using a UV-activated derivative of Re595 LPS (ASD-Re595 LPS) in cross-linking assays, we demonstrated a critical role of MD-2 and TLR4 carbohydrates in LPS cross-linking to the LPS receptor. The ability of the various glycosylation mutants to support cell activation was also evaluated in transiently transfected HeLa cells. The double mutant of MD-2 failed to support LPS-induced activation of an interleukin-8 (IL-8) promoter-driven luciferase reporter to induce IL-8 secretion or to activate amino-terminal c-Jun kinase (JNK). Similar results were observed with TLR4 mutants lacking three or more N-linked glycosylation sites. Surprisingly, the reduction in activation resulting from expression of the Asn mutants of MD-2 and TLR4 can be partially reversed by co-expression with CD14. This suggests that the functional integrity of the LPS receptor depends both on the surface expression of at least three proteins, CD14, MD-2, and TLR4, and that N-linked sites of both MD-2 and TLR4 are essential in maintaining the functional integrity of this receptor.

A family of related genes encoding the 10 known Toll-like receptors (TLR) is involved in innate immune responses in mammals (1–4). Engagement of TLRs by specific products of the pathogen, i.e. bacterial endotoxin or lipopolysaccharide (LPS) (5–7), bacterial DNA (8), outer membrane lipoproteins and lipopeptides (9–11), and bacterial flagellin protein (12), results in activation of innate immune responses. Among these substances LPS is considered to be the prototypic activator (13, 14). The functional membrane receptor for LPS is comprised of at least three proteins, CD14, TLR4, and MD-2. Binding of LPS to TLR4 and MD-2 is enhanced by CD14 and the plasma protein LPS-binding protein (LBP) (15).

Although there have been a number of detailed structure-function analyses for both CD14 and LBP (16–22), only limited information is available for MD-2 or TLR4. The mature human MD-2 and TLR4 sequences deduced from cDNA contain 160 and 839 amino acids residues, respectively. Both proteins have potential N-linked glycosylation sites. Sequence analysis suggests MD-2 has two potential N-linked glycosylation sites, whereas the ectodomain of TLR4 contains nine. Herein we describe the properties of MD-2 and TLR4 mutants lacking the potential N-linked glycosylation sites. We compared the mutated proteins with the parental forms for cell surface expression for the ability of the expressed proteins to bind LPS using an LPS-cross-linking assay (23) and for their ability to support LPS-induced cell activation. In its totality, the data reported here show that binding of LPS to TLR4-MD-2 and cell activation is dependent on the close association of LPS with TLR4-MD-2, that the function of these two proteins is strongly influenced by the presence of specific N-linked sites, and that CD14 plays in role in effective presentation of LPS to TLR4-MD-2.

MATERIALS AND METHODS

Cell Culture and Transfection—Human embryonic kidney 293 cells and human epithelial HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin. Cells were transfected in 12-well plates (2 × 10^5/well) or in 6-well plates (5 × 10^5/well) with LipofectAMINE/Reagent Plus (Invitrogen). The total amount of DNA was kept constant by supplementing pFLAG-CMV1 vector DNA (Eastman Kodak Co.).

Reagents—Murine anti-human TLR4 monoclonal antibody HTA125 was from Dr. K. Miyake. Re595 LPS and LBP were prepared as described (24, 25), M2 anti-FLAG monoclonal antibody was from Sigma, polyclonal rabbit anti-Myc antibody was from Upstate Biotechnology (Lake Placid, NY), and protein A-Sepharose was from Amersham Biosciences, Inc.

Mammalian Expression Constructs and Site-directed Mutagenesis—Human CD14 cDNA was cloned in pBR/RSV vector as described (26). Myc-CD14 was generated by the addition of a Myc sequence at amino terminus of CD14 into the pCMV5 plasmid. A 220-nucleotide fragment of the leader sequence precedes an NH2-terminal FLAG epitope. Carboxyl-terminal Myc-tagged MD-2 was cloned by introducing a BamHI-SmaI fragment into pALTER (Promega, Madison, WI). FLAG-JNK1 was expressed from a pCMV5 plasmid. A 220-nucleotide fragment of the human IL-8 promoter (nucleotides 1308–1526 in GenBank™ accession number X61850) was inserted into the pALTER vector to generate a plasmid with an IL-8 promoter (pALTER-IL8).
number M28130) was amplified from genomic DNA by PCR using the primers 5’-ATCCCTGGAACTTGTGCTATC-3’ and 5’-TACCCT-TCACACAGGCCTGCAAATT-3’. The Xhol/HindIII fragment, containing the promoter and transcriptional start, was subcloned in pGL3 vector (Promega, Madison, WI). Asn residue mutants were constructed by site-directed mutagenesis with the overlapping extension method by PCR using wt TLR4 or MD-2 DNA templates. Oligonucleotides were designed to replace Asn residues with Ala residues at positions Asn273, Asn305, Asn309, Asn397, Asn456, Asn473, and Asn504 in human TLR4 DNA and at positions Asn26 and Asn114 in human MD-2 DNA. Single-site mutations were first constructed. Multiple-site mutations were constructed progressively. The nomenclature used for the mutants is the following: mut 1.1, TLR4 N526A; mut 1.2, TLR4 N173A; mut 1.3, TLR4 N35A; mut 1.4, TLR4 N282A; mut 1.5, TLR4 N309A; mut 1.6, TLR4 N497A; mut 1.7, TLR4 N575A; mut 1.8, TLR4 N624A; mut 1.9, TLR4 N526A, N173A, N309A; mut 2.1, TLR4 N35A, N282A, N309A, N497A, N575A; mut 2.2, TLR4 N35A, N173A, N309A, N282A, N497A, N575A; mut 2.3, TLR4 N35A, N173A, N282A, N309A, N497A, N575A, N624A; mut 2.5, TLR4 N35A, N173A, N282A, N309A, N497A, N575A, N624A; mut 2.6, TLR4 N35A, N173A, N282A, N309A, N497A, N575A, N624A, N205A; mut 2.7, TLR4 N35A, N173A, N282A, N309A, N497A, N575A, N624A, N205A, N351A; mut 2.8, TLR4 N35A, N173A, N282A, N309A, N497A, N575A, N624A, N205A, N351A, N504A; mut 2.9, TLR4 N35A, N173A, N282A, N309A, N497A, N575A, N624A, N205A, N351A, N504A, N456A. The nucleotide sequences were all confirmed by DNA sequencing.

 crops 1995 Cross-linking reaction:—293 cells cultured in 10-cm Petri dishes were transfected and harvested 2 days after transfection. Cells were washed twice with 50 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM EDTA, pH 7.4, and twice with Dulbecco’s modified Eagle’s medium containing 50 mM Hepes, pH 7.4. Cells were pelleted and resuspended in 100 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium phosphate, pH 7.2, for 10 min in the presence of 1% 2-deoxyglucose and 0.5% SDS. The cell lysate was added to 500 μl of protein A-Sepharose beads and mixed with 30 μg of M2 monoclonal antibody for 2 h at room temperature. Beads were washed three times with lysis buffer, and the washed beads were resuspended in 100 μl of Laemmli buffer and boiled for 10 min. Aliquots from 20 μl of the original mixtures were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. Filters were blocked with 5% nonfat milk in blocking buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated with anti-FLAG antibody for 2 h and with peroxidase-conjugated secondary antibody for 1 h at ambient temperature. Specific bands were revealed using the ECL Plus system (Amersham Biosciences, Inc.). Aliquots of 80 μl of immunoprecipitates were separated on 12% SDS-PAGE. The gels were dried, and incorporated 125I was revealed and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Enzymatic Deglycosylation of MD-2 and TLR4—293 cells were transfected and harvested 2 days after transfection. Cells lysates were prepared, immunoprecipitated with anti-FLAG antibody, and separated into aliquots. Immune complexes were boiled in 50 mM sodium phosphate, pH 7.2, for 10 min in the presence of 1% 2-mercaptoethanol and 0.5% SDS. N-Linked carbohydrates were removed by treatment with 50 units/ml peptide N-glycosidase F, Flavobacterium meningosepticum, N-acetyl neuraminidase (Streptococcus pneumoniae, Roche Molecular Biochemicals). Reactions were performed for 2 h at 37 °C in a final volume of 50 μl.

Flow Cytometry Analysis—Cells were plated at a density of 5 × 10⁶ cells/well in 6-well plates and transfected with the indicated plasmids together with pEGFP-N3 vector (CLONTECH Laboratories, Inc., Palo Alto, CA) for 2 days. Subsequently, cells were harvested, washed twice in phosphate-buffered saline containing 1% fetal calf serum and 0.2% NaN₃, and incubated with anti-FLAG or HAT-125 (10 μg/ml each) for 45 min at 4 °C. After 2 washes, cells were labeled for 45 min with phycoerythrin-conjugated goat ant-mouse antibody (BD Pharmingen, San Diego, CA). Cells were then washed twice and analyzed with a FACS-Calibur flow cytometer (Becton Dickinson, Mountain View, CA).

Reporter Assays—HeLa cells were plated at a density of 1 × 10⁶ cells/well in 12-well plates and transfected with 50 ng of IL-8 promoter-driven luciferase reporter and 10 ng of indicated plasmids. A pCMV-β-galactosidase control plasmid (50 ng) was used for normalizing transfection efficiencies. 18 h after transfection, cells were stimulated with Ref595 LPS for 6 h. Cells were lysed, and lysates were assayed for luciferase activity using reagents from Promega. Luciferase activity was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). For β-galactosidase assays, an aliquot of the cell extracts was incubated in 200 μl of Z buffer (100 mM sodium phosphate, pH 7.5, 10 mM KCl, 1 mM MgCl₂, 50 mM β-mercaptoethanol) and 40 μl of 4 mg/ml O-nitrophenyl β-D-galactopyranoside at room temperature for 20 min. The reaction was stopped by adding 300 μl of 1 M Na₂CO₃, and β-galactosidase activity was measured at A₅₄₀ nm. Each experiment was repeated at least twice.

IL-8 ELISA—The concentration of IL-8 in the culture supernatants of IL-8 Transfected HeLa cells was measured by ELISA using 96-well poly-

RESULTS

MD-2 and TLR4 Contain N-Linked Carbohydrates—The cDNA sequence for MD-2 predicts a protein with a mass of 16 kDa. However, when lysates from transfected 293 cells expressing FLAG-MD-2 were immunoprecipitated using anti-FLAG antibody and analyzed by SDS-PAGE and Western blot, we observed that MD-2 migrates as a protein triplet of –26, 20, and 16 kDa (Fig. 1A, lane 1). It is important to note that the 26-kDa form of MD-2 is found just below a band appearing in all lanes (Fig. 1, A and B) with a slightly slower mobility; this is the antibody light chain present in the immunoprecipitates. To determine whether the apparent heterogeneity of MD-2 results from glycosylation of MD-2 on N- or O-linked sites, the following experiment was performed. 293 cells were transfected with FLAG-MD-2, and immunoprecipitates were prepared with anti-FLAG antibody as described and either left untreated or treated with endoglycosidase H, peptide N-glycosidase F, or β-1,2-mannotetraosylceramide. These results further support our contention that there are N- and/or O-linked carbohydrates on MD-2.

To further support this contention we analyzed the MD-2 amino acid sequence and found two asparagines (Asn) within potential consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr). These Asn residues were replaced with Ala; single mutation (Asn273→Ala) or Asn114→Ala produced decreased amounts of 16 kDa (Fig. 1B, lanes 3 and 4), whereas mutation of both residues resulted in expression of a protein with an apparent mass of 16 kDa (Fig. 1B, lane 5). The 26-kDa form observed with wild-type MD-2 disappeared with each of the three mutants examined. It is interesting to note that overexpression of MD-2Δ114A resulted in a predominant band of 20 kDa, suggesting that this mutant is entirely monoglycosylated. These results further
Asn114 were probed by SDS-PAGE and Western blot analysis with anti-FLAG antibody. 

Peptide N-glycosidase F (PNGase F), or O-glycosidase and analyzed by SDS-PAGE and Western blot analysis using anti-FLAG antibody. B, MD-2 carries N-linked carbohydrates at positions Asn26 and Asn114, 293 cells were transiently transfected with vectors encoding wt MD-2, MD-2Asn26A, MD-2Asn114A, MD-2Asn26A, Asn114A, or empty vector pFLAG-CMV1. Anti-FLAG immunoprecipitates of cell lysates were probed by SDS-PAGE and Western blot analysis with anti-FLAG antibody.

support our contention that MD-2 has two major N-linked glycosylation sites; we have identified these sites as Asn26 and Asn114.

Similarly, to evaluate the contribution of glycosylation to TLR4, FLAG immunoprecipitates from lysates of 293 cells transfected with FLAG-TLR4 were analyzed by SDS-PAGE before and after glycosidase treatment. The recombinant FLAG-TLR4 has an apparent mass of 120 kDa; this is reduced by ~30 kDa in the presence of endoglycosidase H (EndoH) and peptide N-glycosidase F (PNGase, Fig. 2A). In contrast, the electrophoretic mobility of TLR4 remained unchanged in the presence of O-glycosidase. These data are consistent with the presence of N-linked carbohydrate chains in TLR4. To identify which of the nine potential sites within TLR4 are glycosylated, the primary sequence was scanned for appropriate Asn residues, and various single and multiple Asn → Ala mutants were generated. All mutant receptors were generated using wild-type TLR4 with a FLAG moiety at the amino-terminal end. Each of the single Asn → Ala mutants had electrophoretic mobilities that were slightly reduced when compared with wt TLR4, suggesting that each site was likely to be substituted (Fig. 2B). Multiple-site mutations of Asn to Ala produced proteins with electrophoretic mobilities consistent with a lower apparent molecular weight. A mutant with all nine Asn residues mutated to Ala had the fastest mobility in SDS-PAGE, which is consistent with the predicted 90-kDa mass of TLR4 protein.

Role of MD-2 and TLR4 Carbohydrates in Cell Surface Expression, Secretion, and Recognition of LPS—MD-2 is a secreted protein that is found at the cell surface via its interactions with TLR4. Thus, we asked whether the cell surface expression of the MD-2 mutants depends on the presence of the N-linked glycosylation sites. 293 cells were transiently transfected with wt or mutant FLAG-MD-2. Flow cytometry analysis using anti-FLAG antibody demonstrated that all mutants were weakly detected at the cell surface of 293 cells (data not shown). The weak surface expression detected by fluorescence-activated cell sorter analysis for wt MD-2 and its mutants is likely to be explained by the lack of co-expressed molecules such as TLR4, which act as binding partners for MD-2. This is supported by our findings that wt and mutant forms of MD-2 are detected in supernatants of transfected cells (Fig. 3A). Interestingly, an additional band of about 30 kDa could be detected in cultured medium but not in cellular extracts of 293 cells transfected with wt MD-2. The exact biochemical differences accounting for the slower migrating form of MD-2 cannot at present be explained. It is interesting to note that although deglycosylated MD-2 protein levels were similar in the presence or absence of TLR4 co-expression in cellular extracts, the amount of wt MD-2 and MD-2 mutants detected in supernatants when co-expressed with TLR4 were dramatically reduced, probably due to the interaction with TLR4 (Fig. 3A, bottom panel). The totality of these data support the contention that MD-2 glycosylation is not crucial for cell surface expression and secretion of MD-2 in the absence or presence of TLR4.

The next series of experiments were performed to determine the role of the N-linked sugars on one aspect of the functional integrity of the LPS receptor complex. We have previously described the use of a radioiodinated, cross-linkable derivative of Re595 LPS (125I-ASD-Re595 LPS) to show that LPS is in close proximity to the protein components of the LPS receptor, CD14, TLR4, and MD-2 (15). This assay was used to evaluate the role of the N-linked glycosylation sites in MD-2 and TLR4 in forming an LPS receptor complex where LPS is in close proximity to these same proteins components. To assess the role of the N-linked glycosylation sites in MD-2, we used 293 cells expressing CD14, TLR4, and either wt or mutants of MD-2 lacking one or both of the Asn residues encoding N-linked sites. Control experiments revealed essentially equivalent levels of expression of wt and the three mutant forms of MD-2 (Fig. 3B, upper panel). Both of the monoglycosylated forms of MD-2 were radiolabeled by Re595 LPS, similarly to what we observed with wt MD-2. (Fig. 3B, upper panel, lanes 2 and 3). In contrast, no binding was detected with the double mutant of MD-2 (Fig. 3B, upper panel, lane 4). In the same experiment we observed that the two monoglycosylated mutants were sufficient to support binding of Re595 LPS to TLR4, although at a somewhat lower level (Fig. 3B, upper panel, lanes 2 and 3). In contrast, no LPS association with TLR4 was detected in the presence of the MD-2 double mutant.

We and others have shown that co-expression of MD-2 with TLR4 enhanced expression of cell surface TLR4 (15, 27, 28). To examine whether complete glycosylation of MD-2 was necessary for this effect, we compared TLR4 expression in the presence of wt and mutant MD-2 (Fig. 4). The expression of TLR4 was identical with all forms of MD-2, supporting the contention that its N-linked carbohydrates are not required for the enhanced cell surface expression of TLR4.

Because the N-linked carbohydrates of membrane and secreted proteins are important protein modifications often involved in processing and cell surface expression, we next determined their role in TLR4 expression. To verify the subcellular localization of TLR4 mutant receptors, their expression was monitored in HeLa cells by flow cytometry analysis using a monoclonal antibody and the anti-TLR4 monoclonal antibody HTA125. Of the 9 single TLR4 mutants, only receptors lacking Asn26 or Asn75 were not detected on the cell surface of HeLa cells. In contrast these proteins could...
FIG. 2. TLR4 is a highly glycosylated protein. A, TLR4 in pFLAG-CMV1 was transiently transfected into 293 cells. 48 h after transfection, cells were lysed, and cell extracts were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were left either untreated or treated with endoglycosidase H (EndoH), peptide N-glycosidase F (PNGase F), or O-glycosidase and analyzed by SDS-PAGE. B, TLR4 carries N-linked carbohydrates at nine potential glycosylation sites. 293 cells were transiently transfected with vectors encoding for wt TLR4 or various deglycosylated mutants. Anti-FLAG immunoprecipitates of cell lysates were probed by SDS-PAGE and Western blot analysis with anti-FLAG antibody. See "Materials and Methods" for nomenclature of TLR4 mutations.

FIG. 3. MD-2 N-linked glycosylations are required for binding of LPS. A, 293 cells were transfected with specified plasmids for 2 days. Cell supernatants were collected, and cells were lysed. Both cell extract and cell supernatants were immunoprecipitated with anti-FLAG antibody and analyzed by SDS-PAGE and immunoblotting using anti-FLAG antibody. B, 293 cells were transfected with vectors encoding for CD14, FLAG-TLR4 in the presence of wt FLAG-MD-2, and MD-2 N26A, MD-2 N114A, or MD-2 N26A N114A and cultured for 2 days. Cells were incubated with 250 ng/ml 125I-ASD-Re595 LPS and 2.5 μg/ml LBP at 37 °C for 10 min and photolyzed for 4 min on ice. Standard M2 precipitates of lysates were separated by SDS-PAGE and analyzed by autoradiography (top panel) or by immunoblotting (bottom panel) using anti-FLAG antibody, indicating expression of FLAG-TLR4 and FLAG-MD2 proteins. The asterisk indicates an additional band present in cell supernatants but not in cell extracts.
be detected by immunoblotting (Table I and Fig. 2). These data suggest that at least two specific mutations result in impaired trafficking to the plasma membrane and suggest that they might be sequestered to the endoplasmic reticulum. None of the multiple Asn mutants generated containing either one of these mutations could be detected at the plasma membrane. Point mutations of the other Asn did not alter the cell surface expression of TLR4, as detected by flow cytometry.

We further studied the role of TLR4 N-linked carbohydrates in binding LPS using the LPS cross-linking assay facilitated with $^{125}$I-ASD-Re595 LPS (15, 23). Table I summarizes all of the experimental results obtained when expression of wt TLR4 and some of the mutant receptors were compared (experimental data not shown). Single mutants that were expressed at the cell surface had the same LPS binding characteristics as wt TLR4. We failed to observe binding of LPS to TLR4$^{N296A}$ and TLR4$^{N297A}$, the two mutations that prevented cell surface expression of TLR4. Although the absence of only one glycan seemed not to affect binding of LPS to TLR4, mutants lacking two or more glycans lost the ability to interact with LPS. For instance, mut 4.1 and mut 5 were unable to cross-link LPS despite expression of the receptors at the cell surface. Surprisingly, loss of TLR4 glycans seemed to also affect binding of LPS to MD-2. Thus, like MD-2, TLR4 glycans seem to play a key role in the binding of LPS to the TLR4-MD-2 complex.

We then asked whether the MD-2 glycans play any role in the formation of a TLR4-MD-2 complex on the cell surface. 293 cells were transfected with Myc-TLR4 in the presence or absence of FLAG-MD-2 and cultured for 1 h after the start of LPS treatment, and dose-dependent, with as little as 1 ng/ml LPS (data not shown). These results show that N-linked carbohydrates are not involved in the interaction of TLR4 with MD-2, and absence of these carbohydrates did not dramatically alter TLR4 conformation. We also tested for an interaction between FLAG-MD-2 and Myc-CD14. We observed a slight constitutive association between CD14 and MD-2 in cells transfected with CD14-MD-2-TLR4. This interaction could be further enhanced in a transient manner in the presence of LPS (Fig. 5B). Interestingly, only monoglycosylated and aglycosylated forms of MD-2 were capable of making a complex with CD14. Similar results were obtained when using monoglycosylated and aglycosylated MD-2 mutants (data not shown). Expression of FLAG-MD-2 mutant forms and Myc-CD14 was monitored in cell lysates by Western blotting.

**MD-2 and TLR4 Oligosaccharides Are Important for LPS Signaling**—We optimized a transient transfection system in HeLa cells to evaluate the expression of various forms of TLR4, MD-2, and CD14 in order to evaluate the function of MD-2 and TLR4 mutants on the ability of the LPS receptor to support cell activation. HeLa cells were transiently transfected with various combinations of CD14, TLR4, and MD-2 together with a luciferase reporter plasmid containing a portion of the human IL-8 promoter, as described under “Materials and Methods.” The same cells were used to measure production of IL-8 protein as well as to measure amino-terminal c-Jun kinase (JNK) activation to further assess cell activation. Cells co-transfected with wild-type TLR4 and MD-2 gained responsiveness to Re595 LPS; co-expression of CD14 potentiated the response (Fig. 6). The addition of Re595 LPS induced up to a 10-fold increase of IL-8 promoter activity (Fig. 7A). A similar pattern of responses was observed when IL-8 production was measured in cell supernatants by ELISA (Fig. 7B). Cell lysates from TLR4-MD-2-CD14-transfected HeLa cells were also examined for LPS-induced JNK phosphorylation by immunoblotting with an anti-phospho-JNK antibody that specifically recognizes the activated form of JNK (Fig. 6C). We observed a significant increase in LPS-induced JNK phosphorylation in HeLa cells overexpressing TLR4-MD-2 in the absence or presence of CD14. No other combination of expressed proteins supported LPS-induced cell activation. JNK phosphorylation is time-dependent, reaching maximal phosphorylation at 30 min and declining 1 h after the start of LPS treatment, and dose-dependent, with JNK activation detectable with as little as 1 ng/ml LPS (data not shown).

We next transfected cDNAs encoding the MD-2 mutants described above with wild-type TLR4 in the presence or absence of CD14. The transfected HeLa cells were exposed to LPS, and the lysates were analyzed for LPS-induced JNK phosphorylation. LPS-induced JNK phosphorylation was identical in cells co-transfected with either wild-type MD-2 or either of the MD-2 single mutants. In contrast we failed to detect JNK phosphorylation in cells expressing MD-2$^{N296A,N114A}$ (Fig. 7A). Surprisingly this latter effect was reversed by co-expression of CD14 (Fig. 7B). We also observed that the double mutant of MD-2 failed to support LPS-induced activation of the IL-8 promoter-driven luciferase reporter (Fig. 8A) or to induce IL-8 secretion (Fig. 8B). A partial response was observed with both single mutants, and as described for studies of JNK activation, the addition of CD14 partially reversed the effects of the mutations.

We also used the HeLa cell system to evaluate the effects of mutation of N-linked sites in TLR4. Using the IL-8 promoter, we noted that none of the single mutations altered the ability of TLR4 to support LPS-induced cell activation (Fig. 9A and data...
not shown). Progressive multiple mutations of Asn resulted in a diminished capacity to support LPS-induced IL-8 promoter activity. For instance, mut 4.1 and mut 5 were totally unresponsive to LPS, although the expressed protein was still present at the cell surface. Similar to what we described above for aglycosyl MD-2, TLR4 mutants with reduced capacity to support cell activation could be compensated for by the co-expression of CD14. For example, mut 4.1 and mut 5 recovered partial signaling activity after exposure to LPS (Fig. 9B). On the other hand, mutants that are not detectable at the cell membrane (all mutants containing a mutation in either Asn526 or Asn575) remained completely inactive even in the presence of CD14, suggesting the absolute requirement for all components of the LPS receptor to be localized on the plasma membrane.

We further confirmed these findings by measuring IL-8 protein in cell-free supernatants activated as described (Fig. 10). Mutants lacking 4 or 5 glycans (mut 4.1 and mut 5) were totally unresponsive to LPS in the absence of CD14. The addition of CD14 helped cells to circumvent the effects of expressing the TLR4 mutants and resulted in enhanced release of IL-8 in medium approaching about 60% of the levels observed with the wild-type protein when optimal amounts of LPS are used (Fig. 10).

**DISCUSSION**

In this study we have investigated the role of N-linked glycans of TLR4 and MD-2. To understand the requirements of MD-2 and TLR4 N-linked glycosylations, we have used site-directed mutagenesis to make Asn→Ala substitutions within potential N-linked glycosylation sites. This strategy allowed us to demonstrate that the presence of oligosaccharides in MD-2 and TLR4 is necessary for a number of biological functions of the LPS receptor complex such as trafficking from intracellular compartments to the surface for maintenance of a LPS receptor where LPS can be brought into close proximity to the essential signaling proteins and for efficient signal transduction leading to cell activation. Of interest are the findings that even when the expression of aglycosyl MD-2 and TLR4 results in a suboptimal form of the LPS receptor, co-expression of CD14 at the...
Asn-linked glycosylation is a major form of co-translational modification in eukaryotic protein synthesis (29, 30). The functional relevance of mature glycosylation varies among receptors. For instance, the solubility of a protein can be significantly affected by carbohydrates groups present on the outer surface of a receptor protein. The sugars also play a role in protein folding and assembly and in cell trafficking. The oligosaccharide moieties can also have dramatic effects on biological properties of a glycoprotein such as ligand binding affinity, signal transduction, immunogenicity, clearance rate, and in protein-protein association.

Nine sites of potential N-linked glycosylations are present within the extracellular domain of TLR4, whereas MD-2 contains only two. Our experiments using glycosidases support the contention that each of potential N-linked glycosylation sites of both TLR4 and MD-2 appear to be used. Treatment of TLR4 and MD-2 with endoglycosidase H and peptide N-glycosidase F reduced protein size to that calculated for the protein backbone. Further support for multiple glycosylation was also provided by site-directed mutagenesis experiments. Although TLR4 migrates as a single band, resulting from glycosylations of many or all potential sites, MD-2 glycosylations give rise to three different molecular species. The existence of three different isoforms for MD-2 is intriguing. Overexpression of wt MD-2 into cells produced a predominant diglycosylated isoform and two monoglycosylated isoforms. A totally aglycosylated isoform also seemed to naturally exist, albeit at a significantly lower amount. The reason for the presence of three MD-2 glycoforms is still unknown but may contribute to as yet unappreciated complexities within the LPS receptor.

In some cases, N-glycosylation is necessary for proper processing and intracellular transport of proteins. For instance, lack of glycosylation in the α subunit of insulin receptor (31), GLUT1 glucose transporter (32), CD4 (33), and angiotensin receptor (34) dramatically impaired proper targeting of proteins to cell surface and resulted in intracellular trapping of partially deglycosylated proteins, probably in the endoplasmic reticulum. Aglycosylated MD-2 resulting from Asn26 and Asn114 mutations remained expressed at the cell surface and could be detected in cell supernatants, demonstrating that
these sugars did not alter either MD-2 trafficking or proper folding of the protein. On the other hand, we report that of the 9 N-linked glycosylation sites in TLR4, 2 residues (Asn 526 and Asn575) play a critical role in cell surface delivery of TLR4. The importance of these two residues might be explained by their proximity to the Cys-rich region and transmembrane region of TLR4. The Cys-rich domain is presumably involved in disulfide bond formation and may be critical in establishing the native conformation of TLR4. However, the absence of glycan at

![Figure 8](image_url)

**FIG. 8.** Aglycosylated MD-2 is unable to activate IL-8 promoter activity and IL-8 secretion in response to LPS in absence of CD14. HeLa cells were transiently co-transfected with MD-2 constructs plus TLR4 and human IL-8 promoter-driven luciferase reporter plasmid and β-galactosidase vector in the absence or presence of CD14. 24 h after transfection, cells were left unstimulated or stimulated with 100 ng/ml Re595 LPS. Cell extracts were prepared 6 h after LPS stimulation and luciferase activity monitored (A). Cell supernatants were harvested 24 h after LPS exposure and assayed for IL-8 production (B).

![Figure 9](image_url)

**FIG. 9.** TLR4 mutants lacking multiple N-linked carbohydrates do not respond to LPS. HeLa cells were transiently co-transfected with TLR4 constructs encoding for deglycosylated mutants plus wt MD-2 and human IL-8 promoter-driven luciferase reporter plasmid and β-galactosidase vector in the absence (A) or presence (B) of CD14. 24 h after transfection, cells were left unstimulated or stimulated with 100 ng/ml Re595 LPS for 6 h. Cell extracts were prepared, and luciferase activity was monitored.

![Figure 10](image_url)

**FIG. 10.** TLR4 deglycosylated mutants do not produce IL-8 in response to LPS. HeLa cells were transiently co-transfected with various TLR4 constructs encoding for deglycosylated mutants plus MD-2 in the absence (A) or presence (B) of CD14. 24 h after transfection, cells were left unstimulated or stimulated with 100 ng/ml Re595 LPS for 24 h. Cell supernatants were harvested and assayed for IL-8 production. Ctrl, control.

Asn624 did not prevent TLR4 processing to the cell surface. Furthermore, our results cannot exclude the possibility that the retention of these mutants in the cell is due to misfolding caused by the introduction of substituted residues rather than to its lack of N-linked carbohydrates. It is also well documented that glycans have a direct effect on the protein folding process. The addition of glycans allows cells to synthesize and secrete more complex proteins. Aglycosylated MD-2 was properly secreted and retained its ability to interact with TLR4. Thus, MD-2 glycans are unlikely to be involved in the proper folding of the protein. Moreover, aglycosylated MD-2 was also found in cell supernatants of cells expressing wt MD-2. Thus, the absence of binding of LPS to aglycosylated MD-2 suggests a direct role of MD-2 sugars in LPS recognition. Finally, another pos-
sible role of MD-2 glycans is that they stabilize the LPS receptor complex and, particularly, the TLR4 conformation. MD-2 oligosaccharides may influence conformation of the TLR4-MD-2 complex and modify the LPS affinity for LPS receptor. Another possibility is that MD-2 sugars are responsible for LPS-induced TLR4 oligomerization, which eventually results in intracellular signaling. It is interesting to note that TLR4 multiple deglycosylation mutants dramatically reduced LPS cross-linking not only to TLR4 but also to MD-2. Altogether, these observations support the contention that TLR4 and MD-2 influence each other’s conformations, which allows recognition of LPS. The presence of N-linked oligosaccharides likely plays a critical role in the stabilization of the receptor complex and/or binding to LPS.

Previous studies indicate an important role for N-linked oligosaccharidic chains in the binding and/or signal transduction properties of several cytokines or hormone receptors. Glycosylation of the insulin and epidermal growth factor receptors appears to be required for ligand binding activity (35, 36). On the other hand, deglycosylation of the CD14 possibly plays a critical role in regulating the phenomenon. Our LPS cross-linking experiments show that glycans from MD-2 and TLR4 are necessary for maximal cross-linking of LPS to both proteins. The absence of the two N-linked carbohydrates from MD-2 abolished LPS binding to MD-2 and subsequent signal transduction. Undoubtedly related to these findings is the correlation between LPS binding to TLR4 and/or MD-2 mutants and LPS sensitivity (IL-8 secretion and JNK activation). We previously reported a maximal association of LPS to TLR4 and MD-2 in the presence of CD14. Nonetheless, a weak association of LPS could be observed in the absence of CD14. This is consistent with CD14-independent cell activation at higher LPS concentrations reported by other groups (41, 42). Here we show that HeLa cells can respond to LPS in the absence of CD14. CD14 was not detectable at the cell surface of HeLa cells by fluorescence-activated cell sorter analysis and reverse transcriptase-PCR. However, it is still possible that soluble CD14 in the serum of cell culture might contribute to LPS signaling through TLR4-MD-2. To address this issue, we performed IL-8 promoter reporter assays in the absence of serum. In data not shown, we observed that serum deprivation of cells did not alter LPS activity, ruling out the absolute requirement of CD14 in LPS signaling in HeLa cells. It has been shown that CD14 is not absolutely necessary for LPS signaling, and alternate receptors for LPS are sufficient to enable responses. For example, peripheral blood mononuclear cells from CD14-deficient mice responded to high concentrations of LPS, suggesting that CD14-independent pathways of LPS activation exist (41). Furthermore, Shimazu et al. (42) report that introduction of TLR4 and MD-2 in the absence of CD14 into Ba/F3 was sufficient to restore LPS responses. This response was enhanced by the addition of CD14. These results, in addition to molecular genetic studies, reported by others, support a direct role of TLR4-MD-2 in the recognition of LPS and, therefore, a role of these two proteins as a receptor for LPS. The weak cross-linking observed in our previous studies is inherent to the difficulty of the LPS binding assays and the sensitivity of the method. Here we provide additional data to support a role for CD14 in LPS presenting LPS to its functional signaling receptor that is comprised of at least TLR4 and MD-2.

Some studies have reported that endotoxin can be detected by intracellular receptors in the cytoplasm of mammalian cells. For instance, epithelial cells are unresponsive to extracellular LPS, and yet LPS can activate NF-kB when directly introduced into cells (43). HS90 has been reported to be able to bind LPS and probably participates in LPS intracellular trafficking from cell membrane to the Golgi reticulum (44). Ran/TC4 is an GTPase important for nuclear transport, and it has been shown that it could restore LPS-mediated proliferation of B cells from C3H/HeJ mice, suggesting a role of Ran in LPS signaling, albeit by undefined mechanisms (45). More recently, Nod1, a leucine-rich repeat-containing protein, has been involved as an intracellular receptor for LPS leading to NF-kB activation (46). However, it is unclear whether these proteins are direct receptors for LPS or if LPS is transferred from plasma membrane to intracellular organelles. In any case, we show that TLR4 needs to be present at the cell surface to transduce intracellular signaling. The mutants containing either N526A or N575A mutations were correctly expressed in cells but were not delivered to the plasma membrane and were unable to bind LPS and, therefore, to subsequently activate HeLa cells.

In summary we have shown for the first time that selected N-linked glycosylation sites of both MD-2 and TLR4 play key roles in the maintenance of the ability of the LPS receptor to recognize LPS and to transduce effective transmembrane signals linked to multiple cell activation pathways. These findings suggest that small molecules that selectively interfere with the oligosaccharides of TLR4 and/or MD-2 might be useful as novel therapeutics in settings where LPS is a causative agent.

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