The Differential Impact of Disulfide Bonds and N-Linked Glycosylation on the Stability and Function of CD14*

Received for publication, September 11, 2007, and in revised form, November 30, 2007 Published, JBC Papers in Press, December 5, 2007 DOI 10.1074/jbc.M707640200

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Innate immunity is the first line defense against invading pathogens. During Gram-negative bacterial infection, the Toll-like receptor 4 and MD-2 complex recognize lipopolysaccharide present in the bacterial cell wall. This recognition can be enhanced 100–1000-fold by CD14. However, the beneficial role provided by CD14 becomes detrimental in the context of sepsis and septic shock. An understanding of how CD14 functions will therefore benefit treatments targeted at both immune suppression and immune enhancement. In the present study, we use site-directed mutagenesis to address the role of disulfide bonds and N-linked glycosylation on CD14. A differential impact is observed for the five disulfide bonds on CD14 folding, with the first two (Cys6–Cys17 and Cys15–Cys32) being indispensable, the third and fourth (Cys168–Cys198 and Cys222–Cys255) being important, and the last (Cys287–Cys313) being dispensable. A functional role is observed for the first disulfide bond because the C6A substitution severely reduces the ability of CD14 to confer lipopolysaccharide responsiveness to U373 cells. Two of the four predicted glycosylation sites, asparagines 132 and 263, are actually involved in N-linked glycosylation, resulting in heterogeneity in CD14 molecular weight. Furthermore, glycosylation at Asn132 plays a role in CD14 trafficking and upstream and/or downstream ligand interactions. When mapped onto the crystal structure of mouse CD14, the first two disulfide bonds and Asn132 are in close proximity to the initial β strands of the leucine rich repeat domain. Thus, disulfide bonds and N-linked glycosylation in the initial β sheets of the inner concave surface of CD14 are crucial for structure and function.

CD14 is myeloid-specific leucine rich repeat (LRR)3 protein expressed abundantly on mature monocytes and macrophages (1–3) and at low levels on some neutrophils (4). CD14 is dubbed a pattern recognition receptor because it recognizes multiple pathogen-related molecules (reviewed in Ref. 5) and some common phospholipids (6–8). The best studied and the most biologically relevant ligand is lipopolysaccharide (LPS, also known as endotoxin) from the Gram-negative bacterial cell wall during infection (3). By facilitating the monomeric dissociation of LPS and downstream interaction with the ultimate LPS receptor, the Toll-like receptor 4 (TLR4), and myeloid differentiation protein 2 (MD-2) complex (9, 10), CD14 heightens the sensitivity of the immune system to infection (3, 5, 11–14). This protective role of CD14, however, becomes devastating in the context of septic shock because it exaggerates an already overactive response. Corroborative evidence comes from the fact that CD14 knock-out mice are LPS-insensitive and resistant to septic shock (15, 16), whereas transgenic mice expressing human CD14 are hypersensitive to LPS stimulation (17). An understanding of how CD14 functions would therefore benefit treatment targeted not only at enhancing innate immunity but also at ameliorating septic shock (18, 19).

The underlying mechanism by which CD14 facilitates LPS recognition remains obscure. It remains unknown whether a conformational change in CD14 is involved or whether CD14 merely acts as a shuttle between LPS and the TLR4-MD-2 complex (13). It remains unknown whether a ternary complex is formed between LPS, CD14 and TLR4-MD-2, and if so, whether CD14 interacts directly with MD-2 or TLR4 in the transferring process. It is unlikely that CD14 would participate directly in the signaling cascade, because direct interactions can be observed between lipid A and TLR4-MD-2 complex (Kd ~ 3 nM) (14). With CD14, LPS can be recognized at extremely low concentrations (0.01–1 ng/ml), ~2–3 orders of magnitude lower than that without (5, 20).

Biochemical studies have identified residues on CD14 essential for LPS binding. By deletion mutagenesis, Juan et al. (21) established that residues 57–64 are important for LPS binding. This region is also protected from limited proteolysis after incubation with LPS (22). Using alanine scanning mutagenesis, Stelter et al. (23) demonstrated that residues 39–44 are important for LPS binding. When mapped onto the crystal structure of mouse CD14, residues 57–64 flank one side of the entrance of a hydrophobic pocket, whereas residues 39–44 reside at the bottom of the hydrophobic pocket (24). Because this hydrophobic pocket is big enough to accommodate the acyl chains of LPS, Lee and co-workers (24) proposed it as the LPS-binding pocket. A similar pocket is observed in the crystal structure of MD-2 determined recently by Satow and co-workers (25). The four acyl chains of lipid IVa, an LPS antagonist, are indeed buried in the pocket in the co-crystal structure (25).
CD14 is a glycoprotein with multiple LRRs. An interesting feature of LRR proteins is that cysteines are interspersed in the repeats forming disulfide bonds stabilizing adjacent repeats (26). All 10 cysteines of CD14 have been implicated in disulfide bonds (27). The detailed pairing patterns are inferred from the crystal structure of mouse CD14 (24). Adjacent cysteines form pairs in the structure except for the first pair, where Cys17 and Cys15 pairs with Cys32 (see Fig. 1a). The last disulfide bond of human CD14 (Cys287–Cys333) was not seen in the crystal structure of mouse CD14 because these two cysteines are absent from the mouse sequence. However, the structural and functional relevance of these disulfide bonds has not been addressed. It remains unknown whether these disulfide bonds are the prerequisite of CD14 folding or the result of physical proximity after CD14 folding.

Four Asn residues have been predicted as the glycosylation sites (N-linked) for human CD14 (see Fig. 3a). However, only two of them (Asn132 and Asn261, which corresponds to Asn263 in human CD14) were seen in the crystal structure of mouse CD14 (24). Another Asn residue, Asn156, which is not conserved between the two species, was also glycosylated in the crystal structure of mouse CD14. Indeed, only these two sites were identified as N-linked glycosylation sites for soluble CD14 present in the plasma using a glycoproteomic approach (28). The biological importance of these two glycosylation sites, however, remains unknown.

In the present study, we used site-directed mutagenesis to address these questions. Each Cys was individually replaced with Ala, and the four Asn residues were mutated to Ala individually or in combination. Our results here demonstrate that disulfide bonds and N-linked glycosylation, which are involved in orienting the initial β strand of the inner concave surface of the LRRs, are critical for the structure and function of CD14.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Construction of Baculovirus Stocks**—The human CD14 genes that encode the signal peptide and mature CD14 with a C-terminal 8-amino acid deletion (construct 348) were produced by PCR (29) using the following primers: the forward primer included a BamHI restriction site, the Shine-Dalgarno sequence, and the Kozak sequence, and the reverse primer included a BamHI restriction site, the EcoRI restriction site, and codons for a six-histidine (H6) tag. The human CD14 CDNA gene engineered on PCDNA3 was used as the template (30). The PCR products were introduced to the pEntr3C vector (Invitrogen) by restriction enzyme digestion (BamHI and EcoRI) and ligation and then transferred to pDest8 vector via site-specific LR recombination (Gateway cloning technology; Invitrogen). The expression vector, pExp348H6, was created to encode precursor CD14 (lacking the last 8 amino acids) and a C-terminal six-histidine tag. The ten cysteines were individually mutated to alanine in pExp348H6 by QuikChange mutagenesis (Stratagene) to produce single mutants C5A, C15A, C17A, C32A, C168A, C198A, C222A, C253A, C287A, and C333A. The four Asn residues, Asn132, Asn263, and Asn304, were replaced with Ala individually and in combination to produce single, double, triple, and quadruple mutants. All of the mutations were confirmed by sequencing of the entire gene.

Bacmids were obtained by transforming the pExp vectors into competent DH10Bac™ *Escherichia coli* cells (Invitrogen) (31). Positive clones were identified by a blue and white screen and were confirmed by PCR using M13 forward and reverse primers. Bacmids were then co-transfected with Cellfectin (Invitrogen) into SF9 cells to produce baculoviruses encoding the desired CD14 constructs. Positive clones were confirmed by Western blotting. The viruses were quantified by plaque assay as per the manufacturer’s instruction (Invitrogen).

**Western Blotting**—SF9 cells were cultured in serum-free medium (SF900 II; Invitrogen) and infected by baculovirus at a multiplicity of infection of 2 during log phase. Three days post-infection, the medium was separated from the cells and clarified by low speed centrifugation (500 × g, 5 min). The cells were then lysed by lysis buffer containing 0.5% Triton X-100 and 10 mM Tris, pH 8.0, for analysis of total protein expression. The medium and lysed cell suspension were then analyzed by SDS-PAGE (32) and transferred to polyvinylidene difluoride membrane (Immunobion-P) using a tank electroblot apparatus (Bio-Rad) for Western blot analysis. Penta-His antibody (mouse IgG1; Qiagen) was used to detect recombinant CD14. Horse-radish peroxidase-conjugated goat anti-mouse antibodies (Bio-Rad) were used as the secondary antibody. The blots were then incubated with horseradish peroxidase substrate (enhanced chemiluminescence substrates; GE Healthcare) and developed by exposure to film (Hyperfilm; GE Healthcare).

**Protein Expression and Purification**—Suspension cultures were used for large scale production of recombinant proteins for selected mutants. Medium (1–2 liters) were collected 3 days post-infection and clarified by low speed centrifugation (500 × g, 5 min), high speed centrifugation (10,000 × g, 30 min), and filtration. Secreted CD14 was concentrated by ammonium sulfate precipitation (75%, w/v), followed by resolubilization in 10 mM Tris buffer, pH 8.0. Concentrated CD14 was purified by nickel-nitrioltriabetic acid (Qiagen) affinity chromatography using 150 mM imidazole to elute the protein. The proteins were further purified on a Hi-trap Q (GE Healthcare) anion exchange chromatography column using a linear gradient of NaCl (from 0 to 1 M in 10 mM Tris, pH 8.0) with a flow rate of 0.5 ml/min at 4 °C. The aggregate state was examined by size exclusion chromatography over a Superdex 200 column (1 × 30 cm; GE Healthcare) run in 10 mM Tris, 150 mM NaCl, pH 8.0, at 4 °C with a flow rate of 0.5 ml/min. Protein signals were monitored by UV absorbance at 280 nm. Molecular weights were estimated from gel filtration standards (Bio-Rad) run under identical conditions. Fractions containing CD14 were concentrated by centrifugation on Amicon Centricron concentrators and buffer-exchanged to 10 mM Tris, pH 8.0, for long term storage at −20 °C or −80 °C. The concentrations were determined by UV absorbance at 280 nm using the extinction coefficient 31010 liters·M⁻¹·cm⁻¹ for all CD14 constructs (33).

**Deglycosylation**—Purified proteins (0.1 mg/ml, 100 μl) were denatured by heating in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) for 10 min. G7 buffer (50 mM sodium phosphate, pH 7.5, 1% Nonidet P-40) and 2500 units of peptide N-glycosidase F (PNGaseF) (New England Biolabs) were added to the samples. The mixtures were then incubated for 1 h at 37 °C. The samples before and after PNGaseF treatment were
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The five disulfide bonds of human CD14 inferred from the biochemical data and the crystal structure of mouse CD14 are shown in panel a. Each cysteine was individually replaced with alanine, and the effect was monitored on protein secretion. The results shown in panel b are for two independent experiments (numbers alone and numbers with prime symbol) from two independent virus stocks generated from two different baculoviruses. The media were separated from cells 3 days post-infection. Labeled gels (12.5% SDS-PAGE) were used for separation. CD14 proteins were detected by Western blotting using a Penta-His antibody. The films were exposed for 5 min for visualization. Lane m, medium; lane c, lysed cell suspension. A wild-type 348 sample was run on each gel as a positive control.

analyzed by 12.5% SDS-PAGE and visualized by Coomassie Blue colloidal staining (29).

CD Spectroscopy—CD spectra were acquired on an Aviv 62DS spectrometer with a temperature-controlled cell. The samples were 0.1 mg/ml protein in 10 mM sodium phosphate buffer at pH 7.5. Far-UV CD spectra were collected at 1-nm intervals from 250 to 190 nm at 4 °C as the average of four scans with a 15-s integration time, in a 2-mm-path length cell. Thermal unfolding data were collected at 217 nm at 1-degree intervals from 20 to 100 °C with 60 s of signal averaging in a 2-mm-path length cell.

Functional Assays—U373 cells were cultured in RPMI 1640 medium (Cellgro) with 10% fetal bovine serum (Cellgro) and 10 μg/ml ciprofloxacin. The next day, the supernatants were removed, and the cells were washed four times with phosphate-buffered saline. Fresh RPMI 1640 medium without serum was then added to the culture. The cells were then stimulated with a fixed concentration of LPS (100 ng/ml, E. coli 0111:B4, Sigma, repurified by phenol chloroform extraction) and decreasing concentrations of CD14 (100-6.25 ng/ml). The cells were also stimulated with LPS alone (100 ng/ml) or CD14 alone (1 μg/ml) as controls. After 6 h of stimulation, the supernatants were removed and saved, and fresh serum-free medium was added to the culture. After overnight incubation, the supernatants were again collected, and IL-6 secretion in both sets of supernatants was measured by enzyme-linked immunosorbent assay as per the manufacturer’s instruction (R & D Systems). The results shown are the mean values of triplicate determinations ± S.D. from three independent experiments.

RESULTS

The Differential Impact of Disulfide Bonds on CD14 Folding—The effects of the 10 cysteine to alanine substitutions (Fig. 1a) were examined on protein secretion by Western blotting. Penta-His antibody that specifically recognizes the C-terminal six-histidine tag was used for detection. Only protein secretion is monitored because the template used to generate these mutants, construct 348, is devoid of the intact GPI linkage signal that normally tethers CD14 to the outer membrane (34). Each mutation was tested in two independently constructed baculovirus vectors to ensure that the results are due to the expressed proteins and not a vector artifact. The amount of each construct secreted into the medium was compared with that retained within the cell.

The C6A substitution (Fig. 1b, lanes 1 and 1’) has no effect on CD14 secretion compared with the wild type 348 construct. A significant portion of the total protein is secreted into the medium (m) while the cell (c) retains a larger portion of the over-expressed construct. Surprisingly, replacement of the disulfide bond partner of Cys6 in the C17A mutant (lanes 3 and 3’) abolishes CD14 secretion. Likewise, both the C15A (lanes 2 and 2’) and C32A (lanes 4 and 4’) substitutions result in complete abrogation of CD14 secretion. Because protein secretion is a good index for protein folding here, these results demonstrate that the first two disulfide bonds (Cys15–Cys32) are essential for CD14 folding. The paradoxical effect of C6A substitution, however, indicates the existence of some salvaging process for this particular mutant.

In comparison, the substitutions from Cys168, Cys198, Cys222, and Cys253 to Ala reduce, but do not completely block, CD14 secretion (Fig. 1b, lanes 5, 5’, 8, and 8’). A significant amount of protein is still detected in the medium for these mutants, indicating that although disulfide bonds formed by these cysteines (Cys168–Cys198 and Cys222–Cys253) contribute to CD14 folding and secretion, they are not essential. Mutations at Cys237 and Cys233 have no effect on protein secretion (Fig. 1b, lanes 9, 9’, 10, and 10’), suggesting that the disulfide bond formed between these two cysteines (Cys232–Cys233) results primarily from physical proximity and is not essential for stability and secretion.

N-Linked Glycosylation Accounts for the Observed Heterogeneity—In examining the gels of the Cys mutants (Fig. 1b), it is clear that the expressed proteins do not run as single bands. The apparent heterogeneity in size could arise either from proteolysis or from post-translational modifications such as glycosylation. Proteolysis at the C terminus is unlikely because the primary antibody used to detect CD14 in Western blotting recognizes the C-terminal six-His tag. N-terminal proteolysis is also unlikely because heterogeneity is still observed when another antibody, 3C10 that specifically recognizes amino acids 7–10 of CD14 (35), is used in Western blotting (data not shown). O-Linked glycosylation is unlikely because galactosamine, essential for O-linkage, is absent from the proteolytic fragments of purified native CD14 (27).

N-Linked glycosylation was examined by treatment of 348 with PNGase F, a glycosidase that hydrolyzes the bond between the innermost GlcNAc and Asn. As shown in lanes 7 and 11 of Fig. 2, deglycosylation by PNGase F completely removes heterogeneity, confirming that wild type CD14 produced in insect cells is glycosylated, and differences in the extent of N-linked glycosylation account for the observed heterogeneity.
glycosylation. In contrast, the substitution of either Asn132 (Fig. 3b, lanes 11 and 11’), 14, 14’, 15, and 15’), respectively. Neither the secretion level nor the apparent heterogeneity of the secreted proteins is affected by the substitutions, indicating that these two sites are not involved in N-linked glycosylation. In contrast, the substitution of either Asn18, Asn304, or both Asn18 and Asn304 with Ala reduces the apparent heterogeneity, confirming that these two sites are indeed involved in N-linked glycosylation in agreement with the crystal structure of mouse CD14. Furthermore, the substitution at Asn132 (Fig. 3b, lanes 12 and 12’) greatly reduces the secretion level, indicating that glycosylation at Asn132 serves as a secretion or sorting signal, not merely a “decoration.” In addition, the apparent migration on SDS-PAGE differs between the two mutants (Fig. 3b, compare lanes 12 and 12’ with lanes 13 and 13’). The migration of the N263A mutant is increased compared with the N132A mutant, indicating that glycosylation at Asn263 results in a longer sugar chain, whereas glycosylation at Asn132 results in a shorter sugar chain. The results are further confirmed by the double mutants that contain either the N132A or N263A mutations. Although no protein is detected in the medium of the double mutant N18A,N132A (Fig. 3b, lanes 16 and 16’), proteins are detected in the medium of the double mutant N18A,N263A (Fig. 3b, lanes 17 and 17’). Furthermore, the bands of the double mutant N18A,N263A are less smeared. Likewise, the secretion level of the double mutant N132A,N304A (Fig. 3b, lanes 19 and 19’)

The Differential Impact of N-Linked Glycosylation on CD14 Secretion—The four asparagine residues, predicted as the N-linked glycosylation sites for human CD14 (Fig. 3a), were mutated to alanines individually or in combination, and the effects on protein secretion were examined by Western blotting using a penta-His antibody. The substitution of Asn18, Asn304, or both Asn18 and Asn304 with Ala reduces the apparent heterogeneity, confirming that these two sites are indeed involved in N-linked glycosylation. Furthermore, the substitution at Asn132 (Fig. 3b, lanes 12 and 12’) greatly reduces the secretion level, indicating that glycosylation at Asn132 serves as a secretion or sorting signal, not merely a “decoration.” In addition, the apparent migration on SDS-PAGE differs between the two mutants (Fig. 3b, compare lanes 12 and 12’ with lanes 13 and 13’). The migration of the N263A mutant is increased compared with the N132A mutant, indicating that glycosylation at Asn263 results in a longer sugar chain, whereas glycosylation at Asn132 results in a shorter sugar chain.

The results are further confirmed by the double mutants that contain either the N132A or N263A mutations. Although no protein is detected in the medium of the double mutant N18A,N132A (Fig. 3b, lanes 16 and 16’), proteins are detected in the medium of the double mutant N18A,N263A (Fig. 3b, lanes 17 and 17’). Furthermore, the bands of the double mutant N18A,N263A are less smeared. Likewise, the secretion level of the double mutant N132A,N304A (Fig. 3b, lanes 19 and 19’) is lower than that of the double mutant N263A,N304A (Fig. 3b, lanes 20 and 20’). The difference in the apparent mobility between the N132A,N304A and N263A,N304A mutants (Fig. 3b, lanes 19 and 19’ versus lanes 20 and 20’) is also consistent with that between the two single mutants that contain either N132A or N263A mutation (Fig. 3b, lanes 12 and 12’ versus lanes 13 and 13’). That is, the apparent molecular weight of N132A,N304A mutant is greater than that of N263A,N304A, further confirming that sugar chains with different lengths are attached to these two sites.

The impact on protein secretion is further confirmed by high order mutants. A low level of secretion is only detected for the triple mutant that does not contain the N132A mutation (Fig. 3b, lanes 22 and 22’), confirming that glycosylation at Asn132 serves as an important sorting signal for CD14 secretion. No secretion is detected for the double (Fig. 3b, lanes 18 and 18’), triple (lanes 21, 21’, 23, 23’, 24, and 24’) and quadruple mutants (lanes 25 and 25’) that contain both the N132A and the N263A mutations.

In summary, our mutagenesis data on the four potential glycosylation sites confirm that Asn132 and Asn263 are involved in N-linked glycosylations of CD14, whereas Asn18 and Asn304 are not. Glycosylation at Asn263 results in a longer sugar chain, and glycosylation at Asn132 results in a shorter sugar chain. In addi-

FIGURE 2. N-Linked glycosylation accounts for heterogeneity. Wild-type 348, and selected mutants (C6A, N263A, and N132A) were purified and treated with PNGaseF to remove N-linked glycosylation. The samples were run on Laemmli gels (12.5% SDS-PAGE) and visualized by Coomassie Brilliant Blue staining. Samples before PNGaseF treatment were run under both non-reducing and reducing (β-mercaptoethanol, BME, 5%) conditions, and samples after PNGaseF treatment (PNGaseF) were run under reducing (β-mercaptoethanol, 5%) conditions only. The protein concentrations were 0.1 mg/ml, and 3-μl samples were loaded to each well. The PNGaseF protein is visible below the CD14 mutants in the lanes treated with PNGaseF. The markers are labeled on the left side in kDa.

FIGURE 3. The impact of Asn to Ala substitutions on protein secretion. The four predicted N-linked glycosylation sites are shown in panel a. The four asparagines were mutated to alanines individually or in combination, and the effect was monitored by Western blotting using penta-His antibody (panel b). Single and double mutants are labeled on the bottom. Triple mutants are labeled by the missing mutation (as indicated by the minus symbol). The quadruple mutant that contains all four mutations is labeled as Quadruple. Two independent experiments (numbers alone and numbers with prime symbol) from two independent virus stocks generated by different baculoviruses were done for each mutant. The media were separated from cells 3 days post-infection. Laemmli gels (12.5% SDS-PAGE) were used for separation. The films were exposed for 1 min for protein visualization. Lane m, medium; lane c, lysed cell suspension. The wild-type 348 sample was run on selective gels as a positive control.
tion, glycosylation at Asn\textsuperscript{132} serves as an important signal for CD14 secretion.

Similar Migration Is Observed on SDS-PAGE for Purified Proteins of Selected Mutants—Three interesting single mutants, N263A, N132A, and C6A, were expressed and purified from insect cells as described under “Experimental Procedures.” Consistent with the results from Western blotting, the migration of N132A and N263A on SDS-PAGE is increased, with (Fig. 2, lanes 7, 9, and 10) or without (Fig. 2, lanes 2, 4, and 5) any reducing reagent. However, both mutants still migrate as two distinct bands, and the difference in the molecular weight of those bands is smaller than differences seen with the wild type protein. This indicates that even at a single site, glycosylation is not homogenous. Deglycosylation by PNGaseF further increases migration (compare lanes 13 and 14 with lanes 9 and 10) and completely removes heterogeneity. In addition, after deglycosylation by PNGaseF, all bands migrate to the same molecular weights (lanes 11, 13, and 14), reconfirming that N-linked glycosylation is responsible for the observed heterogeneity as well as the difference in the apparent molecular weights.

Unexpectedly, the migration of C6A is decreased (Fig. 2, lanes 2 and 3), even under reducing conditions in the presence of 5% β-mercaptoethanol (Fig. 2, lanes 7 and 8). Different from the two Asn mutants and the wild type protein, the bands (Fig. 2, lanes 3 and 9) are smeared. No distinct bands can be picked up on SDS-PAGE with or without reducing reagent. In addition, deglycosylation by PNGaseF (Fig. 2, lanes 8 and 12) increases migration and reduces heterogeneity. However, even after PNGaseF treatment, the band is not as sharp as the other mutants (compare lane 12 with lane 11 and lane 13 with lane 14), and the apparent molecular weight of C6A is still greater than wild type protein or any of the two Asn mutants, indicating that mechanisms other than N-linked glycosylation account for the observed heterogeneity and the increased molecular weight.

Secreted CD14 Proteins Are Primarily Monomeric—The aggregation state of the purified mutants was examined by gel filtration chromatography (Fig. 4). A very sharp peak corresponding to the monomeric state is observed for N132A, indicating that N132A is monomeric like wild type 348. Relatively sharp peaks at monomer size are observed for N263A and C6A, indicating that these two constructs are also primarily monomeric. C6A has the largest shoulder peak at the dimer position, which is not surprising because the free cysteine resulting from the mutation can form intermolecular disulfide bonds. Fractions corresponding to monomeric species of each construct were collected for further biophysical and functional studies.

Secreted 348 Mutant Proteins Are Structured—Far-UV CD spectra were collected for these mutants to examine the effect of the mutations on protein secondary structure. The far-UV CD spectra of the three mutants are very similar to the wild type protein (Fig. 5a). All have a minimum at 215 nm and a maximum near 197 nm, characteristic of proteins composed mainly of β sheets. The broadness of the peak at 215 nm, however, indicates the presence of some α helical structure.

The thermal unfolding transition of CD14 is actually an aggregation process as judged by the dynode voltage and fluorescence scattering. The apparent unfolding temperature, i.e. the midpoint of transition, however, gives us a rough estimate of how the protein stability is affected by the individual mutations. Interestingly, the N132A mutation does not have any effect on protein stability (Fig. 5b), because the apparent unfolding temperature remains the same after the mutation. In comparison, the N263A substitution decreases the unfolding temperature, indicating reduced protein stability. Thus, glycosylation at Asn\textsuperscript{263} stabilizes CD14, which could result from electrostatic interactions introduced by the negative charge of the sugar chain. The C6A substitution, however, increases CD14 stability, which is really surprising, because disulfide bonds are expected to stabilize proteins, and this mutation disrupts one disulfide bond.

The C6A and N132A Mutations Distinctively Regulate CD14 Function—Soluble CD14 confers LPS responsiveness to CD14-negative cells such as U373 cells, and the responses can be conveniently followed by cytokine (e.g. IL-6) production. The activities of these mutants were therefore compared in U373 cells using a fixed concentration of LPS (100 ng/ml) and decreasing concentrations of CD14 (100-6.25 ng/ml). The cells are also stimulated with LPS (100 ng/ml) alone, or CD14 (1 μg/ml) alone as controls to determine serum contamination in the assay and LPS contamination in purified CD14, respectively. Supernatants were collected after 6 h of stimulation, and fresh, serum-free-medium was added to the cells. After another 15 h of incubation, the supernatants were again collected, and IL-6 secretion in both sets of supernatants was compared by enzyme-linked immunosorbent assay.

At the 6-h stimulation time (Fig. 6a), wild type CD14 (348, black filled squares) demonstrates a clear dose-response curve. When the cells are stimulated with 10 times as much 348 (1 μg/ml) without added LPS, the response is negligible, indicating the absence of LPS contamination. A similar dose-response curve is found for N263A (Fig. 6a, open triangles), indicating that glycosylation at Asn\textsuperscript{263} has little effect on CD14 function.
The higher activity at lower doses may arise from low levels of LPS contamination, because the stimulation of N263A at 1/\textmu g/ml without LPS (the last lane) results in significantly higher activities than 348 and the other mutants.

On the contrary, the C6A mutation (Fig. 6a, open diamond) substantially impairs the function, indicating that even though C6A is normally secreted, the disruption of the first disulfide bond between Cys6 and Cys17 impairs the function. The first two disulfide bonds are therefore indispensable for the correct structure and intact function of CD14. In comparison, the activity of N132A (Fig. 6a, open circle) is decreased compared with 348 and N263A, indicating that glycosylation at Asn132 contributes somewhat to the intact function of CD14, but it is probably not essential.

Similar results are observed when the second set of supernatants was used for the measurement of IL-6 production (Fig. 6b). N263A has a dose-response curve similar to wild type CD14 (construct 348), and N132A has intermediate activity, whereas C6A has severely impaired activity. These results further confirm that glycosylation at Asn263 has no impact on CD14 function, whereas glycosylation at Asn132 contributes to LPS recognition or downstream interaction. The first disulfide bond is indispensable for CD14 function.

**DISCUSSION**

Much has been done on CD14 in the past 20 years, culminating in the 90s when CD14 was discovered as the LPS receptor by Wright et al. (3). The lack of a signal transduction region, however, led researchers to propose an alternative receptor that transduces LPS signal to the cytosol, which ultimately lead to the discovery of TLR4 as the major receptor (9, 10) and MD-2 as the co-receptor critical for LPS recognition (10). The role of CD14, exemplified by a 100–1000-fold increase in LPS sensitivity, underscores the importance of addressing some fundamental yet unanswered questions about CD14, among which are the sites and impact of co- and post-translational modifications.
Mutagenesis Studies of Human CD14

The crystal structure of mouse CD14 (24) reveals a deep, N-terminal hydrophobic pocket that has been suggested as the LPS-binding site, because residues implicated in LPS binding for human CD14 flank this pocket in the crystal structure (21–24, 35). This hypothesis is further supported by the co-crystal structure of MD-2 with an LPS inhibitor, lipid IVa, recently determined by Satow and co-workers (25). A similar pocket is observed in MD-2 (25), and the four acyl chains of lipid IVa are indeed buried in the pocket, interacting extensively with the hydrophobic side chains of MD-2 within the pocket (25).

The crystal structure of mouse CD14 also sheds light on the sites of co-translational modifications of human CD14. Five disulfide bonds are implicated from the biochemical studies (27). When mapped to the crystal structure of mouse CD14 (Fig. 7b), the first two disulfide bonds are found in the β sheets in the inner concave surface (the “core” structure), whereas the third and fourth disulfide bonds (Cys168–Cys198 and Cys222–Cys253) are in the loops and helices on the outer surface (the “peripheral” structure). The last disulfide bond (Cys287–Cys333) is not seen in the structure, because the construct used for crystallization has the truncation of C-terminal 33 amino acids, and Cys287 is a tyrosine in the mouse CD14 sequence.

Using mutants that replace individual cysteines with alanine, we observe a stepwise effect indicating a differential impact of these disulfide bonds on CD14 folding. The first two disulfide bonds (Cys6–Cys17 and Cys15–Cys32; Fig. 1a) are essential for CD14 folding, because the substitution of these cysteines with Ala, except C6A, completely abolishes CD14 secretion. The secretion of C6A, however, suggests the presence of some salvaging process that spares this mutant from misfolding or mistargeting. A plausible explanation lies in the relative position of cysteines Cys6, Cys15, Cys17, and Cys32 in the crystal structure (24) (Fig. 7a). Cys6 forms the first disulfide bond with Cys17, and Cys15 forms the second disulfide bond with Cys32. However, these two disulfide bonds are physically very close to each other, with Cys17 and Cys32 in the middle. The disruption of any of these four cysteines could therefore lead to mispairing between the remaining cysteines. This mispairing of disulfide bonds could result in the lack of secretion observed. The C6A mutation is not as catastrophic because with this mutation, Cys15 or Cys17 can still form a disulfide bond with Cys32, whereas Cys15 and Cys17 cannot form a disulfide bond while still maintaining their β-strand conformation. Therefore, the second and third β strands can still be oriented correctly, which seems essential for maintaining the structure.

However, this interesting mutant, C6A, which escapes the quality control system of the eukaryotic expression system, is biologically inactive. Its ability to confer LPS responsiveness to U373 cells is severely impaired, indicating that even though the first β strand might not be critical for CD14 folding and secretion, it is critical for ligand interaction. These critical interactions could be solely with LPS but may also involve upstream interactions with LPS and/or downstream binding with TLR4+MD2. Furthermore, the migration of C6A on SDS-PAGE is significantly different from wild type protein and the other two mutants even under reducing conditions. The apparent mobility of C6A is higher than wild type protein, even after complete deglycosylation by PNGaseF, suggesting that other post-translational modifications may account for the increased molecular weight. The first disulfide bond is therefore essential for the structural and functional integrity of CD14.

FIGURE 7. The crystal structure of mouse CD14. Ribbon views of the crystal structure of mouse CD14 (Protein Data Bank code 1WWL) (24) are shown in panels a and b. Two molecules (green and blue) as seen in the crystal structure are shown in panel b. Disulfide bonds are highlighted in yellow sticks (panel a) or spheres (panel b), with cysteines (panel a) or pairs (panel b) labeled. Sugar chains are shown in magenta as sticks in panel b. The red arrow in panel b indicates the view for c. The surface potential (panel c) was calculated for one of the two molecules seen in the crystal structure. Red represents negatively charged surface, blue is positively charged surface, and white is noncharged surface. The two regions implicated in LPS binding are shown in cyan (residues 57–64) and green (residues 37–44) in panel c. Asn132 is shown in yellow. The graphics were created in MolMol (panels a and c) and PyMol (panel b).
The third and fourth disulfide bonds (Cys\textsuperscript{168}–Cys\textsuperscript{198} and Cys\textsuperscript{222}–Cys\textsuperscript{253}) contribute to but do not determine CD14 folding, because the substitutions of these cysteines with alanines decrease but do not abrogate CD14 secretion. The last disulfide bond (Cys\textsuperscript{187}–Cys\textsuperscript{313}) has no effect on CD14 folding, because the substitutions of these two cysteines with alanines do not affect CD14 secretion. This is not surprising because the deletion of the C-terminal 33 amino acids of CD14 does not affect secretion (34).

Our mutagenesis data suggest that disulfide bonds Cys\textsuperscript{6}–Cys\textsuperscript{17} and Cys\textsuperscript{15}–Cys\textsuperscript{32} are involved in orienting the first few LRR modules and are essential for CD14 folding. In comparison, disulfide bonds involved only in orienting the peripheral \(\alpha\) helices and loops (Cys\textsuperscript{168}–Cys\textsuperscript{198} and Cys\textsuperscript{222}–Cys\textsuperscript{253}) contribute to CD14 folding, but they are not essential.

The roles of N-linked glycosylation have been widely documented; one is to stabilize the structure, illustrated by correct trafficking, and the other is to maintain the function, such as ligand recognition (36–46). The role of the N-linked glycosylation of human CD14, however, has not been evaluated. The 15 NA mutants were therefore created in an effort to address both the glycosylation pattern and their associated biological relevance.

Substitutions of Asn\textsuperscript{18} and Asn\textsuperscript{304} with Ala do not affect either the secretion level or the apparent heterogeneity of the secreted proteins. Both N18A and N304A migrate as two bands just like the wild type 348. In contrast, mutations at Asn\textsuperscript{132} and Asn\textsuperscript{263} greatly reduce the heterogeneity of the secreted protein. Both N263A and N132A still migrate as doublets on SDS-PAGE, but the difference between them is greatly reduced. Thus, heterogeneity seen in wild type CD14, as well as in the N18A and N304A mutants, is due to glycosylation at Asn\textsuperscript{132} and Asn\textsuperscript{263}. In addition, glycosylation at these two sites results in sugar chains with different lengths. A longer sugar chain is attached to Asn\textsuperscript{263}, and a shorter sugar chain is attached to Asn\textsuperscript{132} for wild type CD14. However, caution should be exercised when interpreting this piece of data, because the length of the sugar chains from insect cells might not represent what happens in mammalian cells (47).

The role of the first two disulfide bonds is clear, because they are directly involved in orienting the N-terminal helices and loops to form the pocket essential for LPS binding. This is confirmed by the loss-of-function by the C6A mutation. The other three mutations cannot be tested for function, because they are not secreted and cannot be purified. In comparison, the effect of glycosylation at Asn\textsuperscript{132} is indirect. The inner concave surface of CD14 is highly, negatively charged (Fig. 7c). The sugar chain is also negatively charged under physiological conditions, introducing repulsive forces against the \(\beta\) sheet and additional repulsive forces between the \(\beta\) strands. The sugar chain is therefore sticking toward to the entrance of the hydrophobic pocket, which could result in slight difference in ligand recognition when this sugar chain is trimmed off. An alternative explanation is that the repulsive force introduced by the sugar chain is responsible for reorienting the nearby \(\beta\) strands, thereby creating a slightly different hydrophobic pocket. Whatever the cause, the net result is a slightly different interface created by the presence or the absence of the sugar chain. Our mutagenesis data illustrate that disulfide bonds and N-linked glycosylation that directly associate with the initial strands of the LRRs are crucial for the structure and function of CD14.

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