Comparison of Lipopolysaccharide-Binding Functions of CD14 and MD-2

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Prior to being recognized by the cell surface Toll-like receptor 4/MD-2 complex, lipopolysaccharide (LPS) in the bacterial outer membrane has to be processed by LPS-binding protein and CD14. CD14 forms a complex with monomeric LPS extracted by LPS-binding protein and transfers LPS to the cell surface signaling complex. In a previous study, we prepared a functional recombinant MD-2 using a bacterial expression system. We expressed the recombinant protein in Escherichia coli as a fusion protein with thioredoxin and demonstrated specific binding to LPS. In this study, we prepared recombinant CD14 fusion proteins using the same approach. Specific binding of LPS was demonstrated with a recombinant protein containing 151 amino-terminal residues. The region contained a hydrophilic region and the first three leucine-rich repeats (LRRs). The LRRs appeared to contribute to the binding because removal of the region resulted in a reduction in the binding function. LPS binding to the recombinant MD-2 was resistant to detergents. On the other hand, the binding to CD14 was prevented in the presence of low concentrations of detergents. In the case of human MD-2, the secondary myristoyl chain of LPS added by LpxM was required for the binding. A nonpathogenic penta-acetyl LPS mutant lacking the myristoyl chain did not bind to MD-2 but did so normally to CD14. The broader LPS-binding spectrum of CD14 may allow recognition of multiple pathogens, and the lower affinity for LPS binding of CD14 allows transmission of captured materials to MD-2.

Gram-negative bacterial infection often causes lethal endotoxic shock. Lipopolysaccharide (LPS) is the major component of the bacterial outer membrane and is the causative agent of shock (27, 35). LPS is recognized by both innate and adaptive immune systems. LPS is composed of O-antigen repeats, the core region, and lipid A. The O-antigen structures vary by strain and are recognized by the adaptive immune system, resulting in the production of specific antibodies. The target structure for the innate immune system is lipid A, which has conserved structures (27). Toll-like receptors (TLRs) play important roles in the recognition of pathogen-associated molecular patterns (PAMPs) (17). TLR4 is the major receptor for LPS and causes intracellular signal transduction (8, 25, 26). Among the TLR family proteins, TLR4 is unique in having an amino-terminal amphipathic region and 10 LRRs (13). A truncated form containing 152 amino-terminal amino acids was demonstrated to have all full functions for LPS recognition (11, 37). This truncated form contained an amino-terminal amphipathic region and the first RP105/MD-1 complex was also found to be involved in the recognition mechanism for LPS (23, 24). In the same way as MD-2, MD-1 was also shown to be required for cell surface expression of RP105 (19, 23). However, the binding of LPS to MD-1 was not detectable (34, 39). In addition to charge interactions, we demonstrated the critical role of the interaction between MD-2 hydrophobic residues and lipid A acyl chains. A nonpathogenic penta-acetylated LPS lacking the secondary myristoyl chain did not induce immunological responses in human cells (3, 30, 31). We found that the mutant LPS did not bind to human MD-2. For the interaction, phenylalanyl residues of lipid A acyl chains of MD-2 played critical roles (34). The basic residues and phenylalanyl residues required for LPS binding were conserved in human and mouse MD-2 but not in MD-1 sequences. To induce cell responses, the hydrophobic acyl chains of LPS must be displayed to MD-2 anchored on the cell surface.

LPS acyl chains are buried in the membrane structure and are not accessible from the outside. Even if bacterial bodies are broken, the structures are still hidden inside the membrane micelle. LPS-binding protein is capable of accessing LPS in the membrane structure (28, 32, 33). LPS-binding protein pulls out LPS (36) and transfers it to CD14. CD14 forms a stable complex with monomeric LPS and displays it to the TLR4/MD-2 complex (5, 18). CD14 exists as two forms: a glycosylphosphatidylinositol-anchored membrane form and a soluble form (41). The extracellular domain contains an amino-terminal amphipathic region and 10 LRRs (13). A truncated form containing 152 amino-terminal amino acids was demonstrated to have all full functions for LPS recognition (11, 37). This truncated form contained an amino-terminal amphipathic region and the first

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three LRRs but lacked the latter seven LRRs. The amphipathic region contained critical residues for LPS binding (4, 10). On the other hand, the function of the three LRRs in the binding is not clear. Deletion of entire LRRs from CD14 resulted in the failure of expression in mammalian cells (11). In a previous study, we prepared functional recombinant MD-2 proteins using a bacterial expression system (34). Using the same system, we prepared a recombinant CD14 protein containing the 151 amino-terminal amino acids. We also obtained a truncated CD14 containing 69 amino-terminal amino acids, which was unsuccessful for expression in a mammalian system (11). To analyze the transfer mechanism of LPS from CD14 to MD-2, we compared MD-2 and CD14 in the LPS-binding function.

MATERIALS AND METHODS

Preparation of LPS. A crude LPS was purified from 1 g of the acetone-dried Origami B (DE3; Novagen) strain derived from Escherichia coli K-12 by the phenol-water method as described previously (2). The material was then dissolved in 10 ml of distilled water containing 0.2% triethylamine and 0.5% sodium deoxycholate (DC). An equal volume of water-saturated phenol was added, and extraction was performed at room temperature for 5 min. The water phase was collected by centrifugation, and sodium acetate and ethanol were added at final concentrations of 30 mM and 75%, respectively. The mixture was chilled at −20°C for 1 h and centrifuged. The pellet was dried and resuspended in 2 ml of water containing 0.2% triethylamine. After dialysis against ultrapure water and freeze-drying, the pellet was weighed. Contamination by proteins and nucleotides was not detectable by electrophoresis followed by ethidium bromide staining or silver staining (40).

Plasmid constructions. A CDNA fragment containing the entire coding region of CD14 was amplified by PCR from Human Leukocyte Marathon-Ready cDNA (Thermo Electron Co., Waltham, MA) at an internal pressure of 15,000 lb/in2. The PCR product was digested with ClaI and NotI and subcloned into pBluescript-KS (Stratagene, Cedar Creek, TX). A CDNA fragment coding the 151 amino-terminal amino acids was amplified from the template using a sense primer, 5′-CGCGATCCCGACCACGCCAGAACCTTGTGAG-3′, and an antisense primer, 5′-CCGGCGGCGGCCGCCTATTATCAAGCGTTGACCGTGTCAGCATAC-3′. The PCR product was digested with ClaI and NotI and subcloned into pET32b vector as described above. The resulting product was subcloned into the pET32b vector as described above. The resulting recombinant protein was composed of thioredoxin (Trx), the CD14 fragment, His tag, S tag, and CD14 fragments from the amino terminus and was designated CD14AA151. A CDNA fragment for the 69 amino-terminal amino acids of CD14 was amplified using the same sense primer and an antisense primer, 5′-GCCAGGACCGATTTCATGCAAGCC-3′, and an antisense primer, 5′-CCGGATCCCGACCACGCCAGAACCTTGTGAG-3′. The PCR product was digested with BamHI and NotI and subcloned into the pET32b expression vector (Novagen, Madison, WI). The nucleotide sequence for the recombinant protein was confirmed by sequencing using a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit with 7-daza-dGTP (Amersham Biosciences, Piscataway, NJ) and an ALF-express sequencer (Amersham Biosciences). The result suggested that the recombinant protein was composed of thioredoxin (Trx), the CD14 fragment, His tag, S tag, and CD14 fragments from the amino terminus and was designated CD14AA151. A CDNA fragment for the 69 amino-terminal amino acids of CD14 was amplified using the same sense primer and an antisense primer, 5′-CCGGCGGCGGCCGCCTATTATCAAGCGTTGACCGTGTCAGCATAC-3′. The PCR product was subcloned into the pET32b vector as described above. The resulting fusion protein was named CD14AA69. The Trx fusion protein with tags produced by the original pET32b vector was used as a control in this study.

Protein expression and purification. Origami B or the BL21(DE3) lprA mutant was transformed with the plasmids and cultured at 37°C until an optical density (A600) of 0.7 was achieved. Proteins were then cultured for 12 to 14 h at 25°C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture fluid was collected and resuspended in a lysis buffer (20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM 2-mercaptoethanol [2-ME], 1 mM phenylmethylysulfonyl fluoride [PMSF], 20 μg/ml DNase, and 100 μg/ml lysozyme) and incubated at 37°C for 30 min. The protein was then collected using a French pressure cell press (Thermo Electron Co., Waltham, MA) at an internal pressure of 15,000 lb/in2. After centrifugation at 12,000 rpm for 5 min, the supernatants were applied on nickel-adsorbed chelating Sepharose (Amersham Biosciences) columns (Ni columns) equilibrated with a solution of 50 mM phosphate buffer, pH 7.8, 0.3 M NaCl, 5 mM 2-ME, 0.1 mM PMSF, and 100 mM imidazole. After washing, adsorbed proteins were eluted with the same buffer, changing the concentration of imidazole to 500 mM. Purification of CD14AA69 was performed essentially with the same buffers but with lower concentrations of imidazole: 50 mM for washing and 300 mM for elution. Eluted proteins were dialyzed against distilled water containing 50 mM NaCl, 5 mM EDTA, and 5 mM 2-ME. The protein concentration was estimated by a protein assay (Bio-Rad, Hercules, CA), and the purity was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (CBB) staining.

Immunoblotting for LPS detection. Protein preparations were fractionated by SDS-PAGE (15% acrylamide gel) and blotted onto an Immobilon-P membrane filter (Millipore Co., Bedford, MA). Detection of LPS was performed by immunoblotting using anti-LPS monoclonal antibody (MAb) WNI 222-5 (Hybcelt Biotechnology, PB Uden, The Netherlands), alkaline phosphatase-conjugated anti-mouse immunoglobulin G (American Qualex, San Clement, CA), and the BCIP/NBT Color Development substrate (Promega Co., Madison, WI).

LPS binding to purified CD14AA151. CD14AA151 was purified by affinity chromatography on a Ni column as described above but with a washing buffer containing 1% Triton X-100 (TX-100). LPS was not detectable by immunoblotting in the preparation (data not shown). One milligram of the recombinant protein was adsorbed to 100 μl of Ni resin and then incubated at 4°C for 1 h with 0.1 μg of LPS in a solution of 50 mM phosphate buffer, pH 7.4, 5 mM 2-ME, and 0.1 mM PMSF. After washing, bound LPS was detected by immunoblotting as described above.

Effects of the other detergents on the binding were also examined. CD14AA151 was resuspended in Ovum Bi, and a soluble fraction containing the recombinant protein was prepared as described above. One milliliter of the fraction was adsorbed to 50 μl of Ni resin and washed with a washing buffer containing different concentrations of Nonidet P-40 (NP-40) or DC. Bound LPS was detected by immunoblotting as described above.

LPS pull-down assay. Labeling of LPS with biotin-LC-ASA (Pierce, Rockford, IL) was performed as described previously (14). A pull-down assay of the recombinant proteins by the labeled LPS was carried out essentially as described previously (39). Biotinylated LPS (10 μg) was adsorbed to 25 μl of streptavidin-agarose (Vector Laboratories, Burlingame, CA) and incubated at room temperature for 1 h with 20 μg of CD14AA151, CD14AA69, or Trx. After washing with phosphate-buffered saline, precipitated recombinant proteins were detected by Western blotting using an anti-His antibody (Amersham Biosciences).

RESULTS

Expression and purification of recombinant CD14 proteins. The CD14 fragment of the 152 amino-terminal amino acids was expressed in mammalian cells and was demonstrated to have an LPS-binding function (11). The fragment was also expressed in E. coli using a pET system. In this case, the recombinant protein was recovered from inclusion bodies.
Refolding was required to demonstrate the LPS-binding function of the recombinant protein. In this study, we used another pET system, the Trx fusion system. An amino-terminal fragment of 151 amino acids was expressed as a fusion protein with Trx in the Origami B strain, which has defects in its reductases. The recombinant protein CD14AA151 was detected as a 35-kDa protein by SDS-PAGE under reducing conditions. Additional 34-kDa staining was detected under nonreducing conditions, suggesting the possible formation of disulfide bonds in the bacterial cells (Fig. 1). Formation of disulfide bonds appeared to contribute to the solubility of the recombinant protein. Most of the protein was recovered from the soluble fraction of each bacterial extract prepared using a French press (Fig. 1, lanes 3 and 7). The recombinant proteins were effectively purified by affinity chromatography on Ni columns. Another recombinant CD14 containing 69 amino-terminal amino acids, CD14AA69, was also effectively solubilized and purified in the same way (data not shown).

**LPS binding to truncated CD14 proteins.** When recombinant MD-2 was expressed in *E. coli*, it was purified as a complex form with LPS from the host bacteria (34). We analyzed the LPS content in the CD14AA151 preparation by immunoblotting using the anti-LPS MAb. LPS was detected in the CD14AA151 preparation but not in control Trx (Fig. 2A and B). LPS bound to CD14AA151 could be removed by washing with the buffer containing 1% Triton (Fig. 2B, lane 2). LPS-free CD14AA151 and Trx were immobilized on Ni resin and were then incubated with purified LPS. After washing, bound LPS was detected by Western blotting. Specific binding of CD14AA151 was detected but not to control Trx (Fig. 2C). Specific binding of CD14AA151 to LPS micelles was also detected in the pull-down assay (39). Biotinylated LPS was absorbed to Avidin beads and was then incubated with the recombinant proteins. After washing, coprecipitated recombinant proteins were detected by Western blotting with an anti-His tag MAb. Specific binding of CD14AA151 to LPS was demonstrated but not with control Trx (Fig. 3). Only slight binding was detected with CD14AA69.

**Effects of detergents on LPS binding to CD14.** The association of LPS with the cell surface TLR4/MD-2 complex has been shown to be resistant to detergents (1). We demonstrated that LPS directly bound to recombinant MD-2 and that the binding was resistant to a detergent, TX-100 (34). Purification of recombinant MD-2 was performed by affinity chromatography on Ni columns. A considerable amount of LPS was detected in the fractions of the recombinant protein when the washing buffer without detergent was used (Fig. 4A). When 0.1% TX-100 was added to the washing buffer, the amount of LPS in the fractions was reduced, but a significant amount of LPS was still detectable (Fig. 4B). The binding was not abolished completely even with 0.5% TX-100 (Fig. 4C). The effects of Triton on LPS binding to CD14AA151 were also examined in the same experiments. LPS binding to the recombinant protein was detected when the washing was done with the buffer without detergent (Fig. 4D).
detergent (Fig. 4D). In contrast with MD-2, LPS binding was completely abolished with 0.1% TX-100 (Fig. 4E). Inhibition was also demonstrated by low concentrations of another nonionic NP-40 or ionic DC (Fig. 5). Compared with MD-2, binding of CD14 to LPS was quite sensitive to detergents.

**Binding of penta-acylated LPS to CD14.** In a previous study, we found that a mutant LPS produced in an lpxM mutant strain lost its ability to bind to human MD-2 (34). The lpxM gene is required for the addition of the secondary myristate chain to lipid A. *E. coli* lacking the gene produces penta-acylated mu-
tant LPS instead of hexa-acylated LPS. CD14AA151 was expressed in an *lpxM* mutant strain, and the recombinant protein was purified on a Ni column. The fractions for the recombinant protein contained a significant amount of the mutant LPS (Fig. 6A). The mutant LPS was not detectable when the washing buffer contained 0.5% Triton (Fig. 6B).

**DISCUSSION**

Recombinant CD14 proteins have been expressed in several different expression systems, including bacterial expression systems (6, 7, 11, 15). Majerle et al. expressed the full-length and truncated forms of the recombinant proteins in *E. coli* using the pET3a system (15). The recombinant proteins were recovered from inclusion bodies, and refolding was required to demonstrate the LPS-binding function. In this study, we succeeded in expressing a soluble functional CD14 recombinant protein in *E. coli*. A set of 152 amino-terminal residues has been shown to be required for the binding function (11). We expressed the 151 amino-terminal residues as a fusion protein with Trx in the Origami strain defective reductases. Formation of disulfide bonds appeared to be successful under the conditions used, as the appearance of the molecular weight of CD14AA151/Trx on SDS-PAGE under nonreducing conditions was smaller than that under reducing conditions. Formation of an appropriate disulfide bond contributed to solubilization of the recombinant protein (Fig. 1). The recombinant fusion protein was capable of LPS binding without refolding (Fig. 2). In a previous study, we prepared functional recombinant MD-2 with the same expression system (34). Expression of the LPS-binding proteins as fusion proteins with Trx in *E. coli* was useful to obtain functional recombinant proteins.

A set of 152 amino-terminal residues has been demonstrated to be required for the LPS recognition mechanism (11). The region is composed of a hydrophilic domain (amino acids 1 to 69) and three LRRs (amino acids 70 to 152) (10). The hydrophilic domain has been demonstrated to contain residues critical for LPS binding (4, 10). On the other hand, the function of the LRRs in the region remains unknown. Recently, the crystal structure of CD14 was determined (12). According to the structure, the residues 70 to 152 are located outside of the predicted binding pocket for LPS. However, the present results suggested a contribution from the region to the binding function. We prepared a fusion protein that contained only the hydrophilic region, CD14AA69. The binding function of this recombinant protein was not detectable (Fig. 3). LRR1 to LRR3 may function as the backbone to support the pocket structure.

An LPS monomer is known to be stabilized as a complex with CD14 (35). Therefore, the interaction between LPS and CD14 could be stable. However, LPS in the complex must be transferred to the TLR4/MD-2 complex. The strong binding of LPS to cell surface TLR4/MD-2 has been demonstrated, and the binding was resistant to detergents (34). We and others have demonstrated the direct binding of LPS to MD-2; therefore, the major contributor to the binding in the complex could be MD-2 rather than TLR4 (16, 34, 38). We next compared the binding function of the recombinant proteins. MD-2 demonstrated resistance to TX-100, but CD14 was quite sensitive to the reagent (Fig. 4). The other detergents also prevented the binding to CD14 with low concentrations (Fig. 5). The strong bond of MD-2 to LPS appeared to be formed by ionic and hydrophobic interactions (34). The affinity of CD14 for LPS binding could be lower than that of MD-2, and different binding affinity appears to enable transmission of LPS from CD14 to MD-2.

Pathogenic hexa-acylated, but not nonpathogenic penta-acylated, LPS bound to human MD-2 (34). Such specificity for the
acyl chains was not detected in the binding function of CD14. Similar binding with the wild type was shown with the mutant LPS to CD14 (Fig. 6). These results indicate that MD-2, rather than CD14, is critical for the definition of LPS pathogenesis. LPS is not the only PAMP recognized by CD14, CD14 also contributes to the recognition of peptidoglycan by TLR2 (9). Instead of tight binding function of MD-2 appears to be more specific for multiple PAMPs. In the case of MD-2, phenylalanine residues at positions 119 and 121 were critical for the binding function (34). Interaction between the hydrophobic residues and the secondary myristate chain may be required for the stable binding of MD-2. Such hydrophobic interactions may be lacking in the binding of CD14. The binding function of MD-2 appears to be more specific and stronger for pathogenic LPS. These different binding function characteristics of CD14 and MD-2 appear to enable effective host responses to pathogens.

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