Regions of mouse CD14 required for Toll-like receptor 2 (TLR2)- and TLR4-mediated activation of NF-κB were studied in transiently transfected 293 cells. Wild-type CD14 enhanced lipopolysaccharide (LPS)-induced NF-κB-dependent reporter activity in cells expressing TLR4/MD-2, and deletion of amino acid regions 35–44, 144–153, and 270–275 impaired the TLR4-mediated activation. Unlike human CD14, mouse CD14 truncated at amino acid 151 lost the activity. Deletion of amino acids 35–44 or 235–243 also abrogated TLR2-mediated activation of NF-κB, whereas mutants lacking amino acids 35–44 or 235–243 and alanine substitution mutants in regions 151–153 and 273–275 were required for the TLR4-mediated activation. Both deletion mutants lacking amino acids 35–44 or 151–153 or 270–275 retained the activity. Deletion and alanine substitution experiments revealed that amino acids 151–153 and 273–275 were required for the TLR4-mediated activation. Deletion and alanine substitution experiments revealed that amino acids 35–44, 151–153, 235–243, and alanine substitution mutations in regions 151–153 and 273–275 were expressed on the cell surface and retained the ability to associate with TLR4. A cross-linking study with photoreactive LPS showed that the labeling intensities to CD14 mutants/TLR4/MD-2 were paralleled by the ability of CD14 mutants to increase TLR4-mediated activation. These results indicate that different regions of mouse CD14 are required for TLR4- and TLR2-mediated activation of NF-κB and suggest that amino acids 35–44, 151–153, 235–243, and 273–275 of mouse CD14 play an important role in LPS binding and its transfer to TLR4/MD-2.

Bacterial lipopolysaccharide (LPS) is a constituent of the outer membrane of the cell wall of Gram-negative bacteria and plays a major role in septic shock in humans (1, 2). Exposure of macrophages to nanogram quantities of LPS results in rapid activation of a number of transcription factors, including NF-κB, which leads to the synthesis of inflammatory cytokines (3). The cell surface molecules that bind to LPS have been extensively studied, and CD14 has been found to be the major receptor (4–6). Recently, Toll-like receptor 4 (TLR4) has been identified as another molecule that transmits LPS signaling into intracellular components (7, 8).

TLR4 is a mammalian homologue of the Drosophila Toll protein, and it was initially recognized as a molecule that increases constitutive NF-κB activity but not LPS-inducible NF-κB activity (9). However, the finding of a novel accessory molecule, MD-2 (7), which confers LPS responsiveness on TLR4, and analyses of TLR4-deficient (10–14) mice have provided strong evidence for involvement of TLR4 in LPS signaling. Although TLR2 was initially recognized as a signaling molecule for LPS (15, 16), analyses of TLR2-deficient mice (14, 17) and modified phenol extraction studies (18, 19) showed that an endotoxic substance(s) other than LPS is responsible for TLR2-mediated signaling.

CD14 is a glycosylphosphatidylinositol-anchored glycoprotein expressed on leukocytes and is the major receptor responsible for the effects of LPS on macrophages, monocytes, and neutrophils (for a review, see Ref. 2). Since CD14 lacks transmembrane and intracellular domains, it is postulated that CD14 presents LPS to its signaling molecule, TLR4 (20). CD14 has also been reported to be a membrane receptor for various bacterial products, such as peptidoglycan and lipoarabinomannan (for a review, see Ref. 21), and TLR2 has been reported to transmit their signalings (14, 22, 22–25). Thus, CD14 plays a critical role in discriminating bacterial products and dividing their signals into TLR4 or TLR2 depending on the nature of the product. We previously reported that lipid A preparations from various Salmonella strains and synthetic Salmonella-type lipid A (compound 516) possess very little stimulatory activity in human macrophages despite being potently active in murine macrophages (26). On the other hand, lipid A preparations from Escherichia coli and synthetic E. coli-type lipid A (compound 506) were equally active in both human and murine macrophages (26). Thus, Salmonella lipid A shows animal species-specific actions. The mechanism of the animal species-specific actions of Salmonella lipid A is still unknown, but it may be attributable to species differences in the CD14 molecules in recognizing these lipid A molecules. Several studies using monoclonal antibodies to CD14 or CD14 deletion and point mutants to identify the structural requirements for human CD14 as a membrane-bound (27–31) or soluble (4, 32–36) receptor for LPS have been performed, and results have shown that the N-terminal region of human CD14 is required for LPS recognition and signal transduction. However, since no structural studies of mouse CD14, especially the regions of the mouse CD14 molecule required for TLR4/MD-2- or TLR2-mediated activation of NF-κB, had ever been performed, in the present study we investigated these regions by transiently transfecting 293 cells as a first step in clarifying the species-specific actions of Salmonella lipid A.

MATERIALS AND METHODS

Cell Culture and Reagents—The human embryonic 293 cell line (obtained from the Human Science Research Resources Bank, Tokyo, Japan) was grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitro-
gen), penicillin (100 units/ml), and streptomycin (100 μg/ml). LPS was prepared from E. coli 055:B5 (H9262)/H11003 LPS (Sigma) that had not been subjected to repurification was dissolved in a 0.2% triethylamine aqueous solution containing 0.5% deoxycholate and mixed with an equal volume of phenol. The phenol phase was then extracted twice with a 0.2% triethylamine aqueous solution containing 0.5% deoxycholate. Next, the phenol phase was then extensively diazylated against purified water, and after vacuum drying, the dried residue was dissolved in purified water and used as the PEX. Antibody against the ELAP tag epitope (antiserum no. 1060) was a kind gift of Dr. Nancy Rice (NCI-Frederick). Anti-mouse CD14 antibody (M-20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Biotinylated LPS was prepared by dialyzing 1 mg of repurified E. coli R3 F653 LPS and 3 mg of PFP-biotin (Pierce) into a mixture in a 0.3 ml of 0.5% deoxycholate. Next, the mixture was incubated overnight at 4°C. A similar LPS response was observed in 293 cells transfected by PCR-mediated mutagenesis. Mouse (mCD14-DAF) or human (MD-2) by the calcium phosphate precipitation method, and 24 h later, cell extracts were prepared by incubating the cells on ice for 10 min with 0.2 ml of a buffer (10 mm HEPES-KOH, 0.5% Nonidet P-40, and 10 mM KCl, pH 7.9) containing a protease inhibitor mix (Roche Molecular Biochemicals). The cell extracts were diluted to 500 μl with PBS containing 0.1% Nonidet P-40 and boiled in an SDS-sample buffer. The supernatant obtained was subjected to SDS-PAGE followed by Western blot analysis.

Detection of CD14 Proteins Expressed on the Cell Surface—Detection of cell surface CD14 was performed as described previously (37) with a slight modification. Briefly, 293 cells were plated in 6-cm dishes and transfected with the plasmids indicated (μg each) by the calcium phosphate precipitation method. After 24 h, the cells were washed twice with PBS and exposed at 15°C for 30 min to 0.5 mg/ml of a membrane-impermeable biotinylation reagent (sulfon-N-hydroxysuccinimide-LC-biotin; Pierce) dissolved in PBS. After stopping the biotinylation by adding 3 ml of the culture medium, cell extracts were prepared with 200 μl of the PBS above. The cell extracts were dialyzed against purified water to obtain the final product. Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)] hexanamidoethyl-1,3-dithiopropionyl LPS (SBED-LPS) was prepared as follows. Repurified E. coli R3 F653 LPS (2.7 mg) was dissolved in 0.8 ml of purified water plus 0.1 ml KOH, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 10 mM pH 7.9, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% SDS and Triton 1%, 0.25% deoxycholate, and 10 mM KCl, pH 7.9) containing a protease inhibitor mix (Roche Molecular Biochemicals). The cell extracts were diluted to 500 μl with PBS containing 0.1% Nonidet P-40, and after adding an anti-mouse CD14 antibody (M-20) and Protein LA-Sepharose (Sigma), the diluted cell extracts were incubated for 1 h at 4°C with rocking. The Sepharose was washed three times with PBS containing 0.1% Nonidet P-40, and after washing the membranes, the signals were visualized with the enhanced chemiluminescence system (Amersham Biosciences).

**Western Blotting**—Western blotting was carried out in the following manner. 293 cells were collected by trypsinization and suspended in 15-cm culture flasks. Western blotting was performed by precipitation followed by SDS-PAGE and electrophoresis according to the manufacturer’s recommendation (Promega).
cals). After adding immobilized streptavidin–agarose, the cell extracts were incubated for 1 h at 4 °C with rocking. The agarose was washed three times with PBS containing 1% Nonidet P-40, 2 mM EDTA, and biotinylated proteins were eluted from the agarose by incubating with 5 mg/ml water-soluble biotin derivative (sulfo-N-hydroxysuccinimide-biotin; Pierce) dissolved in a buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). After the addition of an anti-mouse CD14 antibody (M-20) and Protein G-Sepharose (Amersham Biosciences), the supernatants were incubated for 1 h at 4 °C with rocking. Finally, the obtained supernatants were boiled in an SDS-sample buffer and subjected to SDS-PAGE followed by Western blot analysis.

RESULTS

Effects of CD14 Deletion Mutants on TLR4- and TLR2-mediated Activation of NF-κB—To explore the structure-activity relationship of mouse CD14, we first examined the effect of wild-type CD14 on TLR4- and TLR2-mediated activation of NF-κB. Human embryonic 293 cells were transfected with an NF-κB-dependent E-selectin (ELAM-1) promoter luciferase reporter gene together with expression plasmids for mouse TLR4 and MD-2 or an expression plasmid for mouse TLR2. The transfected 293 cells were stimulated either with 0.1 ng/ml LPS and 1 μg/ml PEX to activate the TLR4-dependent pathway and TLR2-dependent pathway, respectively. Stimulation with LPS did not increase reporter activity in cells expressing TLR4 and MD-2, whereas LPS markedly increased reporter activity in cells co-expressing mouse CD14 (Fig. 1A). PEX did not increase reporter activity in cells expressing TLR2 either, whereas it markedly increased reporter activity in cells co-expressing mouse CD14 (Fig. 1B).

Next, we examined the effects of CD14 deletion mutants on TLR4- and TLR2-mediated activation of NF-κB. When mutants d35–44, d144–153, d235–243, and d270–270, in which the corresponding amino acid regions were deleted, were co-expressed in 293 cells together with TLR4 and MD-2, little or no increase in reporter activity was observed in response to LPS, but LPS-induced activation was partially retained with d244–247 and fully retained with d312–320 (Fig. 1A). Both d35–44 and d235–243 lost the ability to PEX-induced reporter activity in 293 cells expressing TLR2. Despite the fact that the d144–153 and d270–275 mutants had lost the ability for the TLR4-mediated activation, they were fully able to increase TLR2-mediated activation (Fig. 1B). Both the d244–247 and d312–320 mutants retained the ability for the TLR2-mediated activation (Fig. 1B).

Effect of CD14 Deletion and Substitution Mutants on TLR4- and TLR2-mediated Activation of NF-κB—The 144–153 and 270–275 amino acid regions of CD14 were further analyzed to identify the structural requirements, because they were selectively required for TLR4-mediated activation. Amino acids 144–153 were analyzed first (Fig. 2A). Two deletion mutants, d144–147 and d151–153, were created, and the LPS-induced reporter activity of 293 cells was examined after expressing these mutants in them together with TLR4 and MD-2. LPS stimulation of cells co-expressing d144–147 showed an increase in reporter activity that was comparable with the activity of cells co-expressing wild-type CD14, whereas no increase in reporter activity was observed in cells co-expressing d151–153. LPS-induced activation was also severely impaired in cells co-expressing a CD14 mutant (151–153A) in which amino acids 151–153 were replaced by alanine, whereas replacement of one of these amino acids with alanine (P151A, G152A, and L153A) or arginine (G152R) had no effect on LPS-induced activation. These results indicate that amino acids 151–153 are indispensable to the ability of CD14 to increase TLR4-mediated activation.

Next, we examined the structural requirement of amino acids 270–275 (Fig. 2B). Co-expression of 270–272A instead of wild-type CD14 in 293 cells expressing TLR4 and MD-2 had no effect on LPS-induced activation of NF-κB, but LPS-induced activation was severely impaired in cells co-expressing 273–275A. Replacement of each amino acid from 271 to 275 with alanine (P271A, K272A, G273A, L274A, and P275A) or arginine (G273R), however, had no effect on LPS-induced activation. These results indicate that amino acids 273–275 are indispensable to the ability of CD14 to increase TLR4-mediated activation.

To know the property of CD14 mutants further, we next examined the concentration-response relationship of LPS in 293 cells expressing CD14 mutants, TLR4 and MD-2 (Fig. 3). When wild-type CD14 was expressed with TLR4 and MD-2, the activation of NF-κB was observed at 0.1 ng/ml and reached to the maximum at 10 ng/ml of LPS. When each of CD14 mutants was expressed instead of wild-type CD14, ~10-fold higher con-
Structural Requirement of Mouse CD14

Fig. 2. A and B, effect of CD14 mutants on TLR4/MD-2-mediated activation of NF-κB in 293 cells. 293 cells were transiently transfected with either control vector (mock; 0.1 μg), wild-type (wt) CD14 plasmid (0.1 μg), or the CD14 mutant plasmid indicated (0.1 μg), together with TLR4 (2 ng) and MD-2 (2 ng) plasmids and pELAM-L luciferase reporter plasmid. After 24 h, cells were either left unstimulated (open columns) or stimulated with 0.1 μg/ml LPS for 6 h (closed columns), and luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in 293 cells expressing wild-type CD14. Values are means ± S.E. from at least three independent experiments.

Fig. 3. Concentration-response relationship of LPS in 293 cells expressing wild-type or mutant CD14 and TLR4/MD-2. 293 cells were transiently transfected with wild-type (wt) CD14 plasmid (0.1 μg) or the CD14 mutant plasmid indicated (0.1 μg), together with TLR4 (2 ng) and MD-2 (2 ng) plasmids and pELAM-L luciferase reporter plasmid. After 24 h, cells were stimulated with the indicated concentrations of E. coli LPS for 6 h, and luciferase activity was measured. Values are means ± S.E. from at least three independent experiments.

Concentrations of LPS in 151–153A and 273–275A, and 100-fold higher concentrations of LPS in d35–44 were required to achieve equivalent responses to the wild type, and their maximal responses were found to be 70–80% of the maximal response of the wild-type. In cells expressing d235–243, the LPS-response was severely impaired, and even 100 ng/ml LPS produced only 40–50% of the activity.

The substitution mutants that lost the ability to increase TLR4-mediated activation were also examined for PEX-induced activation of NF-κB (Fig. 4). The increase in reporter activity in response to PEX observed in 293 cells expressing TLR2 and wild-type CD14 was also observed in cells expressing 151–153A or 273–275A instead of wild-type CD14 (Fig. 4).

Cell Surface Expression of CD14 Mutants—Next, we attempted to determine whether the CD14 mutants examined above are expressed on the cell surface. The cell surface proteins of 293 cells expressing each of the CD14 mutants were labeled by exposing them to a membrane-impermeable biotinylation reagent, and the biotinylated proteins were collected by precipitation with a streptavidin gel and assayed for CD14 by Western blotting (Fig. 5). In the absence of biotinylation, no CD14 was detected in the precipitate (lane 1, upper panel), although CD14 was expressed (lane 1, lower panel). When CD14 was not expressed, no signals were detected in either the precipitate or the lysate even when biotinylation was performed (lane 2). When the CD14 mutants indicated in Fig. 5 were expressed, all of them were detected in both the precipitates and the lysates (lanes 3–8). These results indicate that these CD14 mutants were expressed on the membrane surface.

CD14 Mutants Are Still Capable of Associating with TLR4—Next, we used a co-immunoprecipitation method to investigate whether the CD14 mutants that had lost TLR4-mediated activity were capable of associating with TLR4. After transfecting 293 cells with wild type or a mutant CD14 plasmid together with a control vector or the plasmid(s) for TLR4 and/or MD-2, CD14 protein was immunoprecipitated from cell extracts prepared from the cells, and co-precipitated TLR4 and MD-2 proteins were detected by Western blotting (Fig. 6A, top panel). No signals corresponding to MD-2 or TLR4 were observed when a control vector or plasmid for MD-2 was co-transfected with the wild-type CD14 plasmid (lanes 1 and 3), but co-precipitation of TLR4 was detected when TLR4 was co-expressed with wild-type CD14 (lane 2). When both TLR4 and MD-2 were co-expressed with wild-type CD14, both TLR4 and MD-2 were co-precipitated (lane 5). When each of the CD14 mutants indi-
detected in Fig. 6 was co-expressed with TLR4 and MD-2, both TLR4 and MD-2 were co-precipitated with every CD14 mutant tested (lanes 5–8). Western blotting of the cell extract showed that all proteins transfected were expressed normally (Fig. 6A, lower panel). These results indicate that these wild-type and mutant CD14 proteins are capable of associating with TLR4 but not MD-2. Thus, we further examined whether these CD14 mutant proteins are capable of associating with TLR4 on the membrane surface. After transfecting 293 cells with wild type or a mutant CD14 plasmid together with a control vector or the plasmids for TLR4 and MD-2, cell surface proteins were cross-linked by a membrane-impermeable bifunctional cross-linking reagent, sulfo-succinimidyl-2-[p-azido-salicylamido]ethyl-1,3-dithiopropionate. This cross-linking reagent possesses amine-reactive N-hydroxysuccinimide in one end and photoreactive hydroxylphenyl azide in the other end with a cleavable 18.9-Å spacer arm. Thus, proteins present in close proximity on the membrane surface are cross-linked to each other with this reagent. After it was cross-linked, hexahistidine-tagged TLR4 was collected by precipitation with nickel-agarose in the presence of 6 M urea to disrupt noncovalent protein-protein association. Then CD14 protein cross-linked with TLR4 was detected by Western blotting after the cross-linking reagent was cleaved with a reducing agent (Fig 6B). When cells were not treated with this cross-linking reagent, CD14 protein was not detected (lane 8), indicating that noncovalently associated CD14 was not co-precipitated with TLR4 in this experimental condition. However, upon cross-linking, all of wild-type and mutant CD14 proteins examined were clearly co-precipitated with TLR4 (lanes 3–7). This result indicates that these wild-type and mutant CD14 proteins are present on the membrane surface in close proximity to TLR4 with a distance of less than 18.9 Å, which is close enough for these proteins to associate with each other. A co-immunoprecipitation experiment was also carried out. After membrane surface proteins were labeled with a membrane-impermeable biotinylation reagent, the biotinylated proteins were collected by precipitation with streptavidin-agarose. Collected proteins were eluted from the agarose by incubating with an excess amount of a watersoluble biotin derivative. Then CD14 was immunoprecipitated from eluted proteins, and co-precipitated TLR4 was detected by Western blotting. The result showed that TLR4 was co-precipitated with all of wild-type and mutant CD14 proteins used in Fig. 6B (data not shown). This result supported the idea that these mutant CD14 proteins are capable
of associating with TLR4 on the membrane surface.

**LPS Binding to Wild-type or Mutant CD14 and TLR4/MD-2**—Next we investigated the binding of LPS to CD14 mutants and the capability of these mutants to transfer LPS to TLR4/MD2 using photoactivable LPS (SBED-LPS). SBED-LPS is a UV-activable cross-linking LPS carrying biotin covalently attached to a photoactivable aryl azide moiety. After transfecting 293 cells with wild type or a mutant CD14 plasmid together with a control vector or the plasmid(s) for TLR4 and/or MD-2, cells were incubated with SBED-LPS. After UV irradiation, proteins cross-linked with SBED-LPS were collected by precipitation with a streptavidin gel. Then cross-linked CD14, TLR4, and MD-2 proteins were detected by Western blotting (upper three panels). Other portions of the cell extracts were assayed for CD14, TLR4, and MD-2 in the same way (cell ext.; bottom panel).

**Fig. 7. LPS binding to wild-type or mutant CD14 and TLR4/MD-2.** 293 cells were transiently transfected with the expression plasmids indicated (3 μg for CD14 and its mutants; 18 μg for TLR4 and MD-2). After 24 h, cells were treated with 100 ng/ml SBED-LPS for 30 min and photolyzed for 4 min. Then cell extracts were prepared and divided into two portions. From one portion of the extracts, proteins cross-linked with SBED-LPS were collected by precipitation with a streptavidin gel. Then cross-linked CD14, TLR4, and MD-2 proteins were detected by Western blotting (upper three panels). Other portions of the cell extracts were assayed for CD14, TLR4, and MD-2 in the same way (cell ext.; bottom panel).

Species Difference in CD14 Molecules—Since N-terminal 151 amino acids of human CD14 were reported to be sufficient to transmit LPS signaling (27, 28), we compared the effects of human (hCD14-DAF) and mouse (mCD14-DAF) TLR4/MD-2 using photoactivable LPS (SBED-LPS). SBED-LPS is a UV-activable cross-linking LPS carrying biotin covalently attached to a photoactivable aryl azide moiety. After transfecting 293 cells with wild type or a mutant CD14 plasmid together with a control vector or the plasmid(s) for TLR4 and/or MD-2, cells were incubated with SBED-LPS. After UV irradiation, proteins cross-linked with SBED-LPS were collected by precipitation with a streptavidin gel. Then cross-linked CD14, TLR4, and MD-2 proteins were detected by Western blotting (upper three panels). Other portions of the cell extracts were assayed for CD14, TLR4, and MD-2 in the same way (cell ext.; bottom panel).

**Fig. 8. Species difference in CD14 molecules.** A, 293 cells were transiently transfected with pELAM-L luciferase reporter plasmid and plasmids for mouse (m; left six columns) or human (h; right six columns) TLR4/MD-2 (2 ng each) together with either control vector (mock; 0.1 μg) or the plasmid for wild-type (wt) CD14 (0.1 μg) or CD14-DAF (0.1 μg) of mouse (m; left six columns) or human (h; right six columns) origin. After 24 h, cells were either left unstimulated (open columns) or stimulated with 0.1 ng/ml E. coli LPS (closed columns) for 6 h, and luciferase activity was measured. Ordinate values are expressed relative to the normalized luciferase activity induced by LPS in 293 cells expressing wild-type CD14. Values are means ± S.E. from at least four independent experiments. B, 293 cells were transiently transfected with the plasmid (0.2 μg each) for wild-type (wt) CD14 or the CD14 mutant indicated of mouse (m) or human (h) origin. After 24 h, cell surface proteins were biotinylated with a membrane-impermeable biotinylating reagent, and cell extracts were prepared. Then biotinylated proteins were collected with streptavidin-agarose. After washing, the agarose beads were boiled in an SDS-sample buffer, and the supernatants were analyzed for CD14 by Western blotting with an anti-EIAV tag antibody.

**Structural Requirement of Mouse CD14**—Next we investigated the binding of LPS to CD14 mutants and the capability of these mutants to transfer LPS to TLR4/MD2 using photoactivable LPS (SBED-LPS). SBED-LPS is a UV-activable cross-linking LPS carrying biotin covalently attached to a photoactivable aryl azide moiety. After transfecting 293 cells with wild type or a mutant CD14 plasmid together with a control vector or the plasmid(s) for TLR4 and/or MD-2, cells were incubated with SBED-LPS. After UV irradiation, proteins cross-linked with SBED-LPS were collected by precipitation with a streptavidin gel. Then collected CD14, TLR4, and MD-2 proteins were detected by Western blotting (Fig. 7). When all of the wild type CD14, TLR4, and MD-2 were expressed, all of these proteins were labeled with SBED-LPS (lane 4). When either TLR4 or MD-2 was omitted, labeling to CD14 was reduced, and no labeling to TLR4 and MD-2 was observed (lanes 2–4), indicating that labeling to TLR4 or MD-2 requires the presence of both TLR4 and MD-2. When CD14 was omitted, no labeling to TLR4 and MD-2 was observed (lane 1), indicating that the labeling to these proteins requires CD14. These results agreed with those described by da Silva et al. (39). When mutant CD14 proteins instead of wild-type CD14 were expressed together with TLR4/MD-2, the labeling to CD14/TLR4/MD-2 was partially observed in cells expressing 151–153A (lane 6) and 273–275A (lane 8), and only trace labeling was observed in d35–44 (lane 5) and d235–243 (lane 7).

Species Difference in CD14 Molecules—Since N-terminal 151 amino acids of human CD14 were reported to be sufficient to transmit LPS signaling (27, 28), we compared the effects of human (hCD14-DAF) and mouse (mCD14-DAF) CD14 truncated at amino acid 151 attached to the GPI anchor sequence of decay-accelerating factor (Fig. 8). When hCD14-DAF was co-expressed in 293 cells together with human TLR4 and MD-2, the increase in NF-κB-dependent reporter activity comparable with wild type CD14 was observed in response to LPS. On the other hand, mCD14-DAF lost the ability to increase LPS-in-
duced reporter activity in 293 cells expressing mouse TLR4 and MD-2 (Fig. 8A), although both hCD14-DAF and mCD14-DAF were expressed on the membrane surface (Fig. 8B).

**DISCUSSION**

We created three types of deletion mutants to investigate the structural requirement of mouse CD14 molecules for TLR4- and TLR2-mediated activations of NF-κB. The first type of deletion mutant (d35–44) lacked the region (amino acids 35–44) that corresponds to the region of human CD14 reported to be important for LPS recognition and signal transduction. The second type of deletion mutant (d144–153, d235–243, and d270–275) lacked regions conserved among species, and the third type of deletion mutant (d244–247 and d312–320) lacked regions that vary among species. We avoided the deletion of possible glycosylation sites of CD14 because they might release the CD14 molecule from the cell membrane by disrupting its GPI anchoring. Deletion of variable regions (d244–247 and d312–320) did not significantly affect either TLR2- or TLR4-mediated activation (Fig. 1, A and B). Deletion of conserved regions (d144–153, d235–243, and d270–275) always caused loss of either TLR2- or TLR4-mediated activation, indicating an important role of these conserved regions in mouse CD14 function. Interestingly, deletion of amino acids 35–44 caused loss of both LPS-induced TLR4-mediated activation and PEX-induced TLR2-mediated activation (Fig. 1, A and B). We used a phenol extract of *E. coli* O111:B4 LPS (PEX) to stimulate the TLR2-dependent pathway, because the LPS preparation repurified by the method described by Hirschfeld *et al.* (18) did not stimulate this pathway (data not shown) as shown by Hirschfeld *et al.* (18). We were also unable to use peptidoglycan and bacterial lipopeptides, which are known to be TLR2 ligands, because peptidoglycan yielded ambiguous results in our experimental system (data not shown), and the TLR2-mediated activity of a synthetic lipopeptide derived from *E. coli* murein lipoprotein, tripalmitoyl-Cys-Ser-Ser-Asn-Ala, was not dependent on CD14 (data not shown). The biochemical nature of the substance(s) contained in PEX is currently unknown. Hirschfeld *et al.* (18) and Dziarski *et al.* (40) reported that bacterial lipoproteins or endotoxin proteins may be responsible for the activation of TLR2-dependent pathway. However, PEX-induced activation of NF-κB was clearly different in terms of CD14 dependence from that induced by bacterial lipoproteins. It is therefore likely that a substance(s) other than bacterial lipoproteins is responsible for the effect of PEX. Our finding that the d35–44 mutant had lost activity in response to PEX suggests involvement of an LPS-like substance(s) in the PEX-induced activation. An effort to identify the substance(s) contained in PEX is currently in progress in our laboratory.

To identify the molecular mechanism responsible for the loss of TLR4-mediated activity by the CD14 mutants, we tested them for membrane surface expression, and the results showed that they were expressed on the cell surface (Fig. 5). Since close interaction between CD14 and TLR4 has been suggested to participate in LPS signaling (20), we investigated the association between CD14 mutants and TLR4, and, as shown in Fig. 6, the results showed that TLR4 was co-precipitated with all mutants (d35–44, 151–153A, d235–243, and 273–275A). Therefore, these regions of mouse CD14 are unlikely to play a significant role in the interaction with TLR4. Our result also suggested that deletion or substitution of these regions does not cause any destructive change in the structure of CD14. In the present study, an association between CD14 and TLR4 was detected by the co-immunoprecipitation technique regardless of MD-2 expression, although our data do not necessarily mean a direct interaction between CD14 and TLR4. Co-precipitation of MD-2 with CD14 was also detected when MD-2 was co-expressed with CD14 and TLR4, but no co-precipitation of MD-2 was detected when CD14 and MD-2 were expressed without TLR4. It is therefore likely that MD-2 was co-precipitated with CD14 through the interaction between MD-2 and TLR4.

The amino acid regions of CD14 between 144–153 and 270–275 were investigated in detail in this study, because these regions were found to be selectively involved in TLR4-mediated activity (Fig. 1, A and B). We were able to narrow these required regions down to 151–153 and 273–275, respectively (Fig. 2, A and B), and since point mutants in which each of the amino acids in these regions was changed to alanine retained TLR4-mediated activity (Fig. 2, A and B), the secondary structure created by these regions is likely to play an important role in the TLR4-mediated activity. By contrast, the amino acid regions between 35–44 and 235–243 were required for both TLR4- and TLR2-mediated activity (Fig. 1, A and B). As stated above, deletion of these regions seems to cause no destructive change in CD14 structure because CD14 mutants (d35–44 and d235–243) in which these regions were deleted retained their ability to associate with TLR4. These regions may play a fundamental role in CD14 function.

In this study, photoactivatable LPS (SBED-LPS) was used to assess LPS binding to CD14 or mutant CD14 and the capability of these mutants to transfer LPS to TLR4/MD2 (Fig. 7). The labeling to TLR4/MD-2 was completely CD14-dependent, because no labeling was observed when CD14 was not expressed. These results agreed with those described by da Silva *et al.* (39). In this experimental condition, we found that labeling intensities to CD14 mutants, TLR4 and MD-2, were roughly paralleled by the ability of CD14 mutants to increase TLR4-mediated activation (see Fig. 3). It is, therefore, likely that the impairment of LPS binding and its transfer to TLR4/MD-2 is involved in the impaired TLR4-mediated activity.

Viriyakosol *et al.* (28), using chimeric CD14 in which amino acids 1–151 of human CD14 were fused to the C-terminal region of decay-accelerating factor, demonstrated that the C-terminal portion of human CD14 beyond amino acid 152 is not required for its role as a membrane receptor for the LPS response. In our study, the amino acid region 273–275 of mouse CD14 was required for TLR4-mediated activation of NF-κB (Fig. 2B). In addition, mouse CD14 truncated at amino acid 151 attached to the GPI anchor sequence of decay-accelerating factor (mCD14-DAF) lost the ability to increase LPS-induced reporter activity in 293 cells expressing mouse TLR4 and MD-2. Therefore, our results demonstrate that human and mouse CD14 are clearly different in terms of the requirement of their C-terminal portions in TLR4-mediated activation of NF-κB. We previously reported that *Salmonella* lipid A exerts very little stimulatory activity on human macrophages despite exerting strong activity on murine macrophages (26). Although similar species-specific actions have been observed with lipid IV₅ (compound 406) and CD14 has been reported (41) not to be involved in its species-specific actions, the structure of lipid IV₅ is not found as a component of any LPS of bacterial origins, and emphasis should be put on the fact that *Salmonella* lipid A is a naturally occurring lipid A. Thus, it will be interesting to investigate the involvement of the C-terminal part of mouse CD14 in species-specific actions of *Salmonella* lipid A.

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REFERENCES

1. Schleuter, J., Heine, H., Ulmer, A. J., and Rietschel, E. T. (1995) Arch. Microbiol. 164, 383–389
2. Ulevitch, R. J., and Tobias, P. S. (1995) Annu. Rev. Immunol. 13, 437–457
3. Hatada, E. N., Krappmann, D., and Scheiderer, C. (2000) Curr. Opin. Immunol. 12, 52–58
4. Juan, T. S., Kelley, M. J., Larson, D. A., Busse, L. A., Hailman, E., Wright, S. D., and Lichenstein, H. S. (1999) J. Exp. Med. 190, 1382–1387
5. Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994) J. Exp. Med. 179, 269–277
6. Wright, S. D., Ramos, B. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1999) Science 284, 1341–1343
7. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) J. Exp. Med. 189, 1777–1782
8. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) J. Biol. Chem. 274, 10689–10692
9. Muzio, M., Natoch, G., Saccani, S., Leverro, M., and Mantovani, A. (1998) J. Exp. Med. 187, 2097–2101
10. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huff, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Leyton, B., and Beutler, B. (1998) Science 282, 2085–2088
11. Vogel, S. N., Johnson, D., Perera, P. Y., Medvedev, A., Larriivre, L., Qureshi, S. T., and Malo, D. (1999) J. Immunol. 162, 5666–5670
12. Qureshi, S. T., Larriivre, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999) J. Exp. Med. 189, 615–625
13. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) J. Immunol. 162, 3749–3752
14. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takeda, H., Ogawa, T., Takeda, K., and Akira, S. (1999) Immunity 11, 443–451
15. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., and Godowski, P. J. (1998) Nature 395, 284–288
16. Kirschning, C. J., Wesche, H., Merril Ayres, T., and Rothe, M. (1998) J. Exp. Med. 188, 2091–2097
17. Heine, H., Kirschning, C. J., Lien, E., Monks, B. G., Rothe, M., and Golenbock, D. T. (1999) J. Immunol. 162, 6971–6975
18. Hirschfeld, M., Ma, Y., Wess, J. H., Vogel, S. N., and Weis, J. J. (2000) J. Immunol. 165, 618–622
19. Tappin, R. I., Akashi, S., Miyake, K., Golenbock, D. T., and Tobias, P. S. (2000) J. Immunol. 165, 5780–5787
20. Jiang, Q., Akashi, S., Miyake, K., and Petty, H. R. (2000) J. Immunol. 165, 3541–3544
21. Ulmer, A. J., Dzierski, R., El-Samalouti, V., Rietschel, E. T., and Flad, H.-D. (1999) in Endotoxin in Health and Disease (Brade, H., Opol, S. M., Vogel, S. N., and Morrison, D. C., eds) pp. 463–471, Marcel Dekker Inc., New York
22. Means, T. K., Lien, E., Yoshimura, A., Wang, S., Golenbock, D. T., and Fenton, M. J. (1999) J. Immunol. 163, 6748–6755
23. Lien, E., Sellati, T. J., Yoshimura, A., Flo, T. H., Rawadi, G., Finberg, R. W., Carroll, J. D., Espevik, T., Ingalls, R. R., Radolf, J. D., and Golenbock, D. T. (1999) J. Biol. Chem. 274, 33419–33425
24. Allergens, A. O., Yang, R. B., Mack, M. B., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P., and Zychlinsky, A. (1999) Science 285, 736–739
25. Scherwieser, D., Dzierski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999) J. Biol. Chem. 274, 17406–17409
26. Tanamoto, K., and Azumi, S. (2000) J. Immunol. 164, 3149–3156
27. Lee, J. D., Kravchenko, V., Kirkland, T. N., Han, J., Mackman, N., Moriarty, A., Leturgy, D., Tobias, P. S., and Ulevitch, R. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9930–9934
28. Viriyakosol, S., and Kirkland, T. N. (1996) Infect. Immun. 64, 653–656
29. Viriyakosol, S., and Kirkland, T. N. (1995) J. Biol. Chem. 270, 361–369
30. Gupta, D., Kirkland, T. N., Viriyakosol, S., and Dzierski, R. (1996) J. Biol. Chem. 271, 23310–23316
31. Stelter, F., Bernheiden, M., Menzel, R., Jack, R. S., Witt, S., Fan, X., Pfister, M., and Schott, C. (1997) Eur. J. Biochem. 243, 100–109
32. Juan, T. S., Hailman, E., Kelley, M. J., Busse, L. A., Davy, E., Empig, C. J., Narhi, L. O., Wright, S. D., and Lichenstein, H. S. (1995) J. Biol. Chem. 270, 5219–5224
33. Juan, T. S., Hailman, E., Kelley, M. J., Wright, S. D., and Lichenstein, H. S. (1995) J. Biol. Chem. 270, 17237–17242
34. Shapiro, R. A., Cunningham, M. D., Ratcliffe, K., Seachord, C., Blake, J., Bajorath, J., Aruffo, A., and Darveau, R. P. (1997) Infect. Immun. 65, 293–297
35. Viriyakosol, S., Mathison, J. C., Tobias, P. S., and Kirkland, T. N. (2000) J. Biol. Chem. 275, 33419–33425
36. Cunningham, M. D., Shapiro, R. A., Seachord, C., Ratcliffe, K., Cassiano, L., and Darveau, R. P. (2000) J. Immunol. 164, 3255–3263
37. Ghazin, T., Murri, M., and Tanamoto, K. (2001) J. Immunol. 167, 3354–3359
38. Murri, M., Murri, Y., Ito, N., Rice, N. R., and Suzuki, T. (1995) J. Endotoxin Res. 2, 337–347
39. da Silva, C., Soldau, K., Christen, U., Tobias, P. S., and Ulevitch, R. J. (2001) J. Biol. Chem. 276, 21129–21135
40. Dzierski, R., Wang, Q., Miyake, K., Kirschning, C. J., and Gupta, D. (2001) J. Immunol. 166, 1938–1944
41. Delude, R. L., Suveda, R. J., Zhao, H., Thieringer, R., Yamamoto, S., Fenton, M. J., and Golenbock, D. T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9288–9292