Extracellular Toll-Like Receptor 2 Region Containing Ser^{40}-Ile^{64} but Not Cys^{30}-Ser^{39} Is Critical for the Recognition of Staphylococcus aureus Peptidoglycan

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Toll-like receptor 2 (TLR2) and CD14 function as pattern recognition receptors for bacterial peptidoglycan (PGN). TLRs and CD14 possess repeats of the leucine-rich motif. To address the role of the extracellular domain of TLR2 in PGN signaling, we constructed CD14/TLR2 chimeras, in which residues 1–356 or 1–323 of CD14 were substituted for the extracellular domain of TLR2, and five deletion mutants of TLR2, in which the progressively longer regions of extracellular TLR2 regions were deleted. PGN induced NF-κB activation in HEK293 cells expressing TLR2 but not in cells expressing CD14/TLR2 chimeras. The cells transfected with a deletion mutant TLR2^{Cys^{30}-Ile^{64}} as well as TLR2^{Cys^{30}-Asp^{160}} and TLR2^{Cys^{30}-Asp^{305}} failed to respond to PGN, indicating the importance of the TLR2 region Cys^{30}-Ile^{64}. Although TLR2^{Cys^{30}-Ser^{39}} conferred cell responsiveness to PGN, the cells expressing TLR2^{Ser^{40}-Ile^{64}} failed to induce NF-κB activation. In addition, NF-κB activity elicited by PGN was significantly attenuated in the presence of synthetic peptide corresponding to the TLR2 region Ser^{40}-Ile^{64}. From these results, we conclude that (1) CD14 cannot functionally replace the extracellular domain of TLR2 in PGN signaling; (2) the TLR2 region Cys^{30}-Ser^{39} is not required for PGN recognition; (3) the TLR2 region containing Ser^{40}-Ile^{64} is critical for PGN recognition.

Gram-positive bacteria, can elicit the excessive release of proinflammatory cytokines from immune cells, resulting in the reproduction of most clinical manifestations of bacterial infections (2–4).

Membrane-bound CD14 (mCD14), a glycosylphosphatidylinositol (GPI)-anchored 55-kDa protein with the peptide of Thr^{1}-Ala^{156} on the surfaces of macrophages and polymorphonuclear leukocytes, serves as a cell-activating receptor for bacterial PGN as well as for LPS (5, 6). CD14 avidly binds PGN as well as LPS (7). CD14 also exists in a soluble form (sCD14) in serum (8). Recombinant sCD14 consisting of the CD14 region Thr^{1}-Gly^{323} has been demonstrated to activate cells in response to LPS (9). Because mCD14 is a GPI-linked membrane receptor but lacks the intracellular signaling domain, signal transducing receptors for LPS and PGN have been postulated.

Toll, originally identified as a regulator for dorsoventral polarity of the Drosophila embryo (10), plays critical functions in the host defense mechanism of the adult fly (11). Toll-like receptors (TLRs), mammalian homologues of Toll, have recently been identified and implicated in signaling by LPS and PGN (12). TLRs, unlike CD14, possess a cytoplasmic domain homologous to that of interleukin-1 receptor (IL-1R). Stimulation by LPS and PGN through TLRs initiates an IL-1R-like NF-κB signaling cascade (13). Genetic approaches have revealed that TLR4 is a signaling receptor for LPS. Pro^{12}→His mutation in Tlr4 gene causes defective response to LPS in C3H/HeJ mice (14). TLR4-knockout mice were hyporesponsive to LPS (15), confirming that TLR4 is a receptor responsible for LPS signaling. Macrophages from TLR2-deficient mice produced pro-inflammatory cytokines in response to LPS but were hyporesponsive to Staphylococcus aureus PGN (16, 17). Recent in vitro studies also demonstrate that PGN-induced cell activation is mediated by TLR2 (18, 19).

Accumulating evidence has revealed that CD14 and TLR2 function as pattern recognition receptors (5, 20). The structural characteristic of these proteins is that they possess leucine-rich repeats (LRRs) (21), which appear to be involved in protein-protein interaction (22, 23). Individual repeats are predicted to contain a short β-strand and α-helix approximately antiparallel to each other (22, 23). CD14 and TLR2 possess 10 and 19 LRRs, respectively. The amino-terminal half of the CD14 molecule, including the region between amino acids 51 and 64 is sufficient for the binding to PGN and LPS (24–27). It remains unknown which region of the amino-terminal extracellular domain of TLR2 is involved in PGN recognition. To address the role of the extracellular domain of TLR2 in PGN signaling, we constructed CD14/TLR2 chimeras, in which mCD14 (residues 1–356) or sCD14 (residues 1–323) was substituted for the extracellular domain of TLR2, and five deletion mutants of TLR2,
in which the progressively longer regions of extracellular TLR2 regions were deleted. This study demonstrates the importance of the amino-terminal region of TLR2 containing Ser40-Ile64 in PGN signaling.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Human embryonic kidney (HEK) 293 cells (obtained from ATCC, CRL-1573) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum. Macrophage-like cell line U937 (JCRB9021) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% fetal calf serum. Peptideoligycan (PNG) from *S. aureus* was obtained from Fluka.

**DNA Constructs**—The 1.3-kb cDNA for human CD14 was obtained as described previously (28). The 2.6-kb cDNA for human TLR2 was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA isolated from U937 cells.

The mutant proteins used in this study are schematically presented in Fig. 1. The mCD14/TLR2 chimera consists of the CD14 region Thr1-Gly323 and the TLR2 region Leu590-Ser784.

### Effects of Synthetic Peptide on PGN-induced NF-κB Activation

1. **Antigen (peptide)**—Peptide (peptide 40–64), SGSSNSPSGLTEAVKSLDLSNRRH, corresponding to the amino acid residues Ser40–Ile64 of TLR2 and a control peptide, GTPVNYTNWYRG, were synthesized by Sigma Genosys Japan and purified by high-performance liquid chromatography. PGN was preincubated at 37°C for 30 min in the presence of 0.1–10 μg/ml peptide 40–64 or control peptide in serum-free medium. After the preincubation, a mixture of the peptide and PGN was added to the wells in which HEK293 cells had been grown on 96-well plates. The plates were incubated for 3 h at 37°C.

2. **Effect of Synthetic Peptide on PGN-induced NF-κB Activity**—The transfection of HEK293 cells was performed with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer’s instructions. Transfection—HEK293 cells were transfected with expression plasmids by FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions.

**RT-PCR**—RT-PCR was performed to confirm mRNA expression in HEK293 cells transfected with the mutant cDNAs. Total RNA was isolated from the cells by using TriZol reagent (Life Technologies, Inc.). A cDNA pool was obtained from 5 μg of RNA using 200 units of Superscript II Reverse Transcriptase (Life Technologies, Inc.) and 0.5 μg of oligo(dt)12–18 primer (Life Technologies, Inc.). PCR amplification was performed with Taq polymerase (Promega) for 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The oligonucleotide primers used for RT-PCR were 5′-GGATCCGAGAAGCCTTATGAG-3′ and 5′-TCCTGATGTCACCTACATGTT-3′ for TLR2 (5′-Cy5-3′, 60°C), 5′-GGATCCGAGAAGCCTTATGAG-3′ and 5′-TCCTGATGTCACCTACATGTT-3′ for TLR2 (5′-Cy5-3′, 60°C), respectively. The PCR products were visualized by staining the gel with ethidium bromide.

**Immunostaining of CD14/TLR2 Chimeras and Fluorescence Microscopy**—Stably transfected HEK293 cell lines expressing CD14, deletion mutants of TLR2 chimera, or sCD14/TLR2 chimera had been grown on polystyrene-coated coverslips. The cells were rinsed with PBS and incubated with 5 μg/ml rhodamine-conjugated concanavalin A (Molecular Probes) in serum-free Dulbecco’s modified Eagle’s medium at 4°C for 15 min. After the incubation, the medium was removed and the cells were fixed in methanol for 4 min at –20°C. The cells were washed with PBS and incubated for 1 h with HRP-labeled goat anti-rabbit IgG (1:500, Santa Cruz Biotechnology) for TLR2. The membranes were finally washed six times with PBS containing 0.1% (v/v) Triton X-100. The proteins that reacted with the antibodies were visualized by using a chemiluminescence reagent (SuperSignal, Pierce).

**NF-κB Reporter Assay**—Activation of NF-κB was measured as previously described (13, 18). HEK293 cells were plated at 1 × 10⁵ cells per well in 24-well plates on the day before transfection. The cells were transiently transfected with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) with 30 ng of an NF-κB reporter construct (pNF-κB-Luc, Stratagene) and 3.5 ng of a constitutively active thymidine kinase promoter (pRL-TK, Promega), together with 150 ng of each transfectant gene of CD14, TLR2, chimeras, and deletion mutants. Forty-eight hours after transfection, the cells were stimulated with 10 μg/ml PGN for 3 h at 37°C.

**Transfection**—The transfected HEK293 cells were washed twice with ice-cold PBS and subsequently washed once with lysis buffer (10 mM Hepes, pH 7.4, containing 1.5 mM MgCl₂ and 10 mM KCl). The cells were scraped with 1 ml of lysis buffer and were transferred into centrifuge tubes. After the centrifugation at 14,000 rpm for 15 min, the pellet was suspended with 40 μl of lysis buffer containing 2.5% (v/v) Nonidet P-40. The suspension was then incubated on ice for 10 min and centrifuged at 14,000 rpm for 10 min. The supernatant containing the cytosolic and membrane proteins was finally collected. Protein concentration was estimated by the bichinonic acid assay (BCA, Pierce) using bovine serum albumin as a standard. 20 μg of the supernatant proteins was subjected to SDS-polyacrylamide (10%) gel electrophoresis by the method of Laemmli (30). The proteins on the gel were electrotransferred onto a polyvinyl difluoride (PVDF) membrane. Blocking of the membrane banding was performed with PNS with PBS containing 3% (v/v) skim milk and 0.1% (v/v) Triton X-100 (blocking buffer) for 30 min. The membrane was then incubated with 10 μg/ml anti-CD14 polyclonal rabbit IgG (28) or 2 μg/ml goat antibody against the TLR2 carboxyl terminus (Santa Cruz Biotechnology, Santa Cruz, CA) for 100 min. After washing three times with the blocking buffer three times, they were incubated for 1 h with HRP-labeled goat anti-rabbit IgG (1:2000, Bio-Rad, Hercules, CA) for CD14 and HRP-labeled donkey anti-goat IgG (1:500, Santa Cruz Biotechnology) for TLR2. The membranes were finally washed six times with PBS containing 0.1% (v/v) Triton X-100. The proteins that reacted with the antibodies were visualized by using a chemiluminescence reagent (SuperSignal, Pierce).

**Recombinant CD14 and PGN Binding**—Recombinant CD14 (Saccharomyces cerevisiae) consisting of the CD14 region Thr1-Gly323 and the six-histidine tag, was expressed in Chinese hamster ovary cells, and the recombinant protein was purified from the culture medium by an affinity column of nickel-agarose beads as described previously (28).

A PGN binding study was carried out by the method described previously for the binding of CD14 to LPS (28). 5 and 20 μg of PGN in 20 μl of ethanol was added onto microtiter wells (Immulon 1B, Dynex),
and the solvent was evaporated in ambient air. After nonspecific binding to the wells was blocked with 10 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 5 mM CaCl2, and 5% (w/v) bovine serum albumin (buffer B), 0–5 μg/ml CD14T1-G323 (50 μl/well) in the buffer B was added and incubated at 37°C for 6 h. The wells were then washed with PBS containing 3% (w/v) skim milk and 0.1% (v/v) Triton X-100 and incubated with anti-CD14 IgG, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. The peroxidase reaction was final

Expression of CD14/TLR2 Chimeras in Transfected-HEK293 Cells—Membrane-bound CD14 (mCD14, residues 1–356) and recombinant soluble CD14 (sCD14, residues 1–323) activate cells in response to bacterial components (5, 9). We constructed two chimeras in which the extracellular domain of TLR2 was replaced with mCD14 (residues 1–356) or sCD14 (residues 1–323) as shown schematically in Fig. 1. The mCD14/TLR2 chimera consists of the CD14 region Met1–Gly323 and the TLR2 region Leu590–Ser784. The sCD14/TLR2 chimera consists of the CD14 region Thr1–Ala356 and the TLR2 region Leu590–Ser784. The sCD14/TLR2 chimera consists of the CD14 region Thr1–Gly323 and the TLR2 region Leu590–Ser784.

To determine whether CD14, TLR2, and the CD14/TLR2 chimeras were expressed in HEK293 cells after transient transfection, mRNA and protein expressions were analyzed by using RT-PCR and immunoblotting. RT-PCR analysis revealed that the PCR products exhibited the expected mobilities of 1221 bp for CD14, 2600 bp for TLR2, 1799 bp for sCD14/TLR2 chimera, and 1898 bp for mCD14/TLR2 chimera (Fig. 2A). The results indicate that mRNAs for these four proteins were expressed in HEK293 cells after transient transfection. The protein expression was also confirmed by immunoblotting analysis with the anti-carboxyl terminus of TLR2 antibody (Fig. 2B) and with anti-CD14 antibody (Fig. 2C).

CD14 and TLR2 exhibited mobilities at ~55 and 90 kDa, respectively, as described previously (5, 32). The CD14/TLR2 chimeras reacted with both anti-TLR2 and anti-CD14 antibodies and exhibited bands at ~70–75 kDa. These results clearly show that the proteins of CD14, TLR2, and CD14/TLR2 chimeras were expressed in transiently transfected-HEK293 cells.

CD14 Cannot Functionally Replace the Extracellular Domain of TLR2 in PGN Signaling—Both CD14 and TLR2, which possess leucine-rich repeats in the extracellular domains, have been proposed to be pattern recognition receptors for PGN (5, 20). CD14 binds PGN (7), and TLR2 confers cell responsiveness to PGN (18). The soluble form of CD14, consisting of the region Met1–Gly323, is biologically active and is capable of binding LPS (9). To investigate the roles of the extracellular domains of CD14 and TLR2 in PGN signaling, we constructed CD14/TLR2 chimeras containing the CD14 region of Met1–Ala356 or Met1–Gly323 and estimated NF-κB activation using luciferase reporter gene assay in transiently transfected HEK293 cells. PGN stimulated NF-κB reporter activity in wt TLR2- but not CD14-transfected cells in a manner dependent upon PGN concentrations (Fig. 3). In addition, cotransfection of CD14 cDNA with TLR2 cDNA enhanced cellular responses by ~43–200%. However, the cells transfected with sCD14/TLR2 chimera cDNA or mCD14/TLR2 chimera cDNA failed to respond to any
the chimeras also failed to confer NF-κB activation in the presence of serum, no cellular activities of measured NF-κB reporter plasmids were detected when stimulated with PGN. Cells were transfected with the indicated expression plasmid vectors together with an NF-κB reporter plasmid (pRL-TK, 3.5 ng). Cells were stimulated with PGN (100–10,000 ng/ml) for 6 h before harvest. The triangles indicate increasing concentrations of PGN (0, 100, 1000, and 10,000 ng/ml). The data shown are mean ± S.E. of three experiments.

**Fig. 3.** PGN-induced NF-κB activation in HEK293 cells expressing mCD14, TLR2, and CD14/TLR2 chimeras. Luciferase activities were determined in 1 × 10^5 HEK293 cells plated in 24-well plates and transfected with the indicated expression plasmid vectors (150 ng) together with an NF-κB reporter plasmid (pNF-κB-Luc, 30 ng) and Renilla luciferase control reporter plasmid (pRL-TK, 3.5 ng). Cells were transfected without the tested plasmids. In these cases none of the cells exhibited significant luciferase activities (C). The data shown are mean ± S.E. of three experiments.

**Fig. 4.** Cell surface localization of CD14/TLR2 chimeras expressed in HEK293 cells. Stably transfected HEK293 cell line expressing CD14 (A–F), mCD14/TLR2 chimera (G–I), or sCD14/TLR2 chimera (J–L) was incubated with rhodamine-conjugated concanavalin A (A, D, G, and J) or control IgG (B), and analyzed using a confocal microscope as described under “Experimental Procedures.” C, F, I, and L are merged images.

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Fig. 5. PGN-induced NF-κB activation in stably transfected HEK293 cell lines. Luciferase activities were determined in stably transfected HEK293 cells (1 × 10^5) plated in 24-well plates and transfected with an NF-κB reporter plasmid (pNF-κB-Luc, 30 ng) and Renilla luciferase control reporter plasmid (pRL-TK, 3.5 ng). Cells were stimulated with PGN (100–10,000 ng/ml) for 6 h before harvest. The triangles indicate increasing concentrations of PGN (0, 100, 1,000, and 10,000 ng/ml). The data shown are mean ± S.E. of three experiments.

Fig. 6. CD14^T1-G323 binds to PGN. A, electrophoretic analysis of CD14^T1-G323. Recombinant CD14 containing the CD14 region Thr^5-Gly^235 (CD14^T1-G235) expressed in Chinese hamster ovary cells was subjected to SDS-polyacrylamide gel electrophoresis under reducing condition. The protein was visualized by Coomassie Blue staining. B, binding of CD14^T1-G323 to PGN. The indicated concentrations of CD14^T1-G323 were incubated with 5 μg/well (open circle) or 20 μg/well (closed circle) PGN coated onto microtiter wells at 37 °C for 6 h. The binding of CD14^T1-G323 to PGN was detected using anti-CD14 IgG as described under "Experimental Procedures." The results show the specific binding that was determined by subtracting the absorbance at each concentration of CD14^T1-G323 when PGN was omitted from that measured in the presence of PGN. The data shown are mean ± S.E. of three experiments.

Fig. 7. Expression of wt TLR2 and TLR2 deletion mutants in transfected HEK293 cells. A, 48 h after transfection with wt TLR2 or each deletion mutant, RNA was isolated from HEK293 cells and RT-PCR for each protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described under "Experimental Procedures." B, the transfected cells were lysed with the buffer containing Nonidet P-40, and the cytosolic and membrane proteins were isolated as described under "Experimental Procedures." 20 μg of the proteins was analyzed by 10% polyacrylamide gel and transferred onto the PVDF membranes. The membranes were probed with anti-TLR2 antibody, and the proteins that reacted with the antibodies were visualized by using chemiluminescence reagent as described under "Experimental Procedures."
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DISCUSSION

The characteristics of mice harboring null allele for TLR2 (16, 17) provide strong in vivo evidence that TLR2 is not involved in the LPS response but is instead involved in cellular responses to PGN. In vitro study with overexpression experiments confirms the essential role of TLR2 in PGN signaling (18). C3H/HeJ mice that have been shown to be hyporesponsive to LPS (14) have been found to possess a missense point mutation within the intracellular domain of TLR4 resulting in the substitution of His712 for Pro. This proline residue is highly conserved among the TLR family, including TLR2. The cytoplasmic domains of TLRs homologous to IL-1R have been demonstrated to be essential for signaling leading to NF-κB activation (13). Because each TLR is involved in signaling for different bacterial ligands (13, 16, 18–20, 33, 34), the extracellular domains of TLRs may define the specificities for various ligands. This study focused on the extracellular domain of TLR2 and investigated the structural requirements of the amino-terminal TLR2 for PGN recognition. The results clearly demonstrate that CD14 cannot functionally replace the extracellular domain of TLR2 in PGN signaling, that the TLR2 region of Cys30-Ser39 is not required for PGN recognition, and that the TLR2 region containing Ser40-Ile64 is critical for PGN signaling.

CD14 serves as a functional receptor for LPS and PGN (5, 7, 35). TLRs have also been demonstrated to be responsible for cellular responsiveness to LPS and PGN (13, 18). Both receptors possess homologous structures consisting of leucine-rich repeats (LRRs) characteristic of a short β-strand and α-helix (23). Although CD14 avidly binds LPS and PGN, CD14 alone cannot confer cell responsiveness to bacterial ligands, because CD14 does not possess the intracellular signaling domain. In addition to membrane-bound CD14 (mCD14), consisting of Thr^1-Ala^56^, the soluble form of CD14 (sCD14) is also able to activate cells in response to bacterial ligands (35). Recombinant sCD14, consisting of the CD14 region Thr^1-Gly^323, has been proved to be active (9). We constructed two chimeric molecules, in which mCD14 (Thr^1-Ala^56^) or sCD14 (Thr^1-Gly^323^) was substituted for the extracellular TLR2 domain (mCD14/TLR2 chimera or sCD14/TLR2 chimera, respectively). Both chimeras were unable to activate the cells in response to PGN regardless of the presence of coexpressed mCD14. Cotransfection of the chimera cDNA with TLR2 cDNA enhanced NF-κB activation when compared with the transfection with TLR2 cDNA alone, indicating that these chimeras possess the same biological activities as mCD14. In addition, the CD14/TLR2 chimeras localized at the cell surface membrane (Fig. 4). These data may rule out the possibility that these chimeras exhibit global defects in...
protein folding. The results indicate the importance of the extracellular TLR2 domain in PGN signaling.

This study has shown that the CD14/TLR2 chimeras cannot activate the cells in response to PGN using the transiently transfected cells and the stably transfected cell lines (Figs. 3 and 5). The results demonstrate that CD14 cannot functionally replace the extracellular TLR2 domain in PGN signaling. We further attempted to differentiate between PGN binding and signaling after PGN binding. To address this issue, we determined whether the CD14 region Thr1-Gly223 (CD14T1-G223) of the extracellular domains of CD14/TLR2 chimeras bind PGN in a cell free assay system, although we were unable to use the whole molecules of the chimeras. CD14T1-G223 avidly bound to PGN (Fig. 6), suggesting that the extracellular domains of the chimeras can bind PGN. In addition, cell surface localization of the chimeric proteins has been demonstrated by immuno-staining with anti-CD14 antibody (Fig. 4). Thus, it is possible to presume that the CD14/TLR2 chimeras can bind PGN but cannot transmit its signals.

We then focused on the TLR2 region of Cys30-Ile64, because the deletion mutant TLR2Cys30-Ile64 as well as the mutants with longer deletion failed to induce NF-κB activation in response to PGN. The further experiments with TLR2Cys30-Ser39 and TLR2Ser140-Ile64 narrowed the critical stretch of primary sequence in the amino-terminal TLR2 region. Because TLR2Cys30-Ser39 conferred significant cell responsiveness, albeit to a lesser extent compared with wt TLR2, it is concluded that the TLR2 region of Cys30-Ser39 is not required for PGN recognition. The TLR2 region of Ser40-Ile64 does not explain all the activity of TLR2 in the recognition of PGN. The TLR2 region of Ser40-Ile64 does not explain all the activity of TLR2 in the recognition of PGN. The TLR2 region of Cys30-Ser39 clearly demonstrates that the cysteine-rich motif (Cys30-Cys65) of TLR2 is not required for PGN recognition.

In conclusion, this study identified a stretch of the sequence of the amino-terminal TLR2 extracellular domain that is required for PGN recognition. The TLR2 region of Cys30-Ser39 is not required, but the region containing Ser40-Ile64 is critical for PGN signaling.

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