Structure-function Relationship of Mycoplasmal Lipoproteins/lipopptides and Their Recognition by Toll-like Receptor 2*

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Abstract: Recent evidence has been accumulated that microbial lipoprotein (LP) plays pathological roles in bacterial infection. The part of LP responsible for the expression of biological activities has been demonstrated to be the N-terminal lipopeptide moiety. Mycoplasmas, wall-less microorganisms, also possess LP capable of activating macrophages or fibroblasts. We have found that M. salivarium LP activates normal human gingival fibroblasts and macrophages to induce production of inflammatory cytokines, and we have purified a 44-kDa LP (LP44) responsible for the activity. In addition, the structure of the N-terminal lipopeptide moiety of LP44 has been determined to be S-(2,3-bispalmitoxypropyl)Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe-Thr-Gly-Trp-Val-Ala-.. The lipopeptide S-(2,3-bispalmitoxypropyl)Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (FSL-1) was synthesized. The lipopeptide FSL-1 activated human gingival fibroblasts and macrophages to produce inflammatory cytokines as LP44 did. Experiments using FSL-1 and its derivatives revealed that the diacylglyceryl and peptide portions of FSL-1 are indispensable for the activation of macrophages and for the recognition by Toll-like receptor 2 (TLR2) and TLR6. We have recently demonstrated that each of several leucine residues located at a leucine-rich repeat of TLR2 play a key role in the recognition of the diacylated lipopeptide FSL-1, mycoplasmal LP and S. aureus peptidoglycan.

Introduction

Invading pathogens are controlled by innate and adaptive immune systems. Adaptive immune system, which is mediated by B and T lymphocytes, recognizes pathogens by rearranged highly specific receptors. However, since the adaptive immune system usually takes several days to eradicate pathogens, more rapid defense mechanisms are provided by innate immunity, which is characterized by the de novo production of mediators that either kill the pathogens directly or induce phagocytic cells to ingest and kill them. It has recently been demonstrated that the innate immune system recognizes invading pathogens through germline-encoded pattern recognition receptors called Toll-like receptors (TLRs) expressed on the surfaces of phagocytic cells such as macrophages and dendritic cells. To date, 10 human TLRs have been identified and have been shown to be critical for signaling by pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan (PGN), lipoprotein (LP)1-5. The activation of innate immunity by TLRs leads to the development of antigen-
specific adaptive immunity. Thus, TLRs control both innate and adaptive immune responses.

Recent evidence has been accumulated that microbial LP plays pathological roles in bacterial infection\(^{1-10}\). *Escherichia coli* LP was first characterized and sequenced by Braun\(^6\), and it has been demonstrated to be biologically active\(^7\). The part of LP responsible for biological activities has been demonstrated to be the N-terminal lipopeptide moiety, the structure of which is S-(2,3-bis-palmitoyloxypropyl)-N-palmitoyl-Cys-Ser-Asn-Asn-Ala\(^{7,12}\). Mycoplasmas, wall-less microorganisms, also possess LP capable of activating macrophages or fibroblasts\(^13\). Mühlradt, et al.\(^13,14\) have identified a 2-kDa lipopeptide called MALP-2 from *Mycoplasma fermentans* that is capable of activating monocytes/macrophages, and they have determined the structure to be S-(2,3-bis-palmitoyloxypropyl)Cys-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Lys. We have also found that *M. salivarium* LP activate normal human gingival fibroblasts (GFh) to induce production of inflammatory cytokines and surface expression of ICAM-1, and we have purified a 44-kDa LP (LP44) responsible for the activity\(^{10}\). In addition, the structure of the N-terminal lipopeptide moiety of LP44 has been determined to be S-(2,3-bis-palmitoyloxypropyl)Cys-Gly-Asp-Pre-Lys-His-Pro-Lys-Ser-Phe-Thr-Gly-Trp-Val-Ala\(^{16}\). The lipopeptide S-(2,3-bis-palmitoyloxypropyl)Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (FSL-1) synthesized on the basis of the N-terminal structure of LP44 showed the same activity as that of LP44\(^{16}\). The framework structure of FSL-1 is the same as that of MALP-2, but they differ in amino acid sequence and length of the peptide portion.

It has already been demonstrated that TLR2 functions as a receptor for microbial LP and lipopeptides\(^{17-35}\), PGN\(^{36-44}\) and glycolipid\(^{45-48}\). Signaling by MALP-2, similar to FSL-1, has been demonstrated to be mediated by TLR2\(^{17,24,27,28}\). Recently, it has been demonstrated that TLR2 requires TLR6 as a coreceptor for recognition of diacylated lipopeptides\(^{24}\). We also found that FSL-1 is recognized by TLR2 and TLR6 as a coreceptor\(^{49,50}\).

Thus, numerous lines of evidence have been accumulated that PAMPs are recognized by TLRs, but it is not clear how TLRs recognize their ligands. We have a great interest in the mechanism by which TLRs recognize PAMPs because we think that elucidation of the mechanisms will provide insight into how microbial infections are controlled. We have recently demonstrated that both diacyl and peptide portion of FSL-1 are recognized by TLR2\(^{50}\) and each of several leucine residues located at a leucine-rich repeat (LRR) of TLR2 are responsible for the recognition of the diacylated lipopeptide FSL-1, mycoplasmal LP and *S. aureus* PGN\(^{50}\).

**Determination of the Structure of N-terminal Lipopeptide Moiety of LP44 and Synthesis of the Diacylated Lipopeptide FSL-1**

LP extracted from the cell membrane of *M. salivarium* was examined for the activation of GFh. The activity was detected in plural LPs, but LP44 responsible for the activity was purified (Fig. 1). The N-terminal amino acid sequence of LP44 was determined to be X-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe-Thr-Glu-Trp-Val-Ala\(-\) by a protein sequencer. Thus, the N-terminal amino acid (X) of LP44 was not determined, because any amino acid peak significantly higher than the others was not observed in the amino acid profile of the first cycle of Edmann degradation. Amino acids after 2nd were easily identified\(^{49}\). This result suggests that the amino group of the N-terminal amino acid is free. Judging from characteristic of Edmann degradation, the N-terminal amino acid is speculated to be cysteine. This is supported by the previous finding that the N-terminal amino acid of lipoproteins from prokaryotes is cysteine, the SH group of which is bound to lipid\(^{31}\). Therefore, the N-terminal amino acid sequence of LP44 was speculated to be Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe-Thr-Glu-Trp-Val-Ala\(-\). It was found by homology search with GeneBank data bases that the amino acid sequence of LP44 had not been report-
Fig. 1  ICAM-1-inducing activity of LP and purification of LP44
A: SDS-PAGE of a protein maker and LP was performed in 10% gel. Lipoproteins separated by SDS-PAGE were transferred to the nitrocellulose membrane. The membrane was cut into 4-mm strips and dissolved in dimethylsulfoxide. Antigen-coated particles were formed by the addition of 50 mM sodium carbonate buffer (pH 9.6) and washed three times with sterile PBS. HGF were stimulated with the particles. ICAM-1 expression was determined by Cell-ELISA.
B: SDS-PAGE of LP44 extracted from SDS-PAGE gels was performed in 10% gels and then LP44 was extracted from the gels according to the method described in Materials and Methods. SDS-PAGE of LP44 extracted and a protein marker was performed in 10% gels and stained with Coomassie brilliant blue.

The effect of proteinase K or lipoprotein lipase on the activation of Gfph by LP44 was investigated and it was found that the activity was resistant to proteinase K and sensitive to lipoprotein lipase, suggesting that the lipid moiety, but not the proteinous moiety, is involved in the expression of the activity. In order to purify the lipid moiety of LP44, LP44 was digested with proteinase K and then fractionated by a reverse-phased HPLC with a linear gradient of isopropanol. The lipid moiety of LP44 was eluted by around 90% isopropanol, because the activity was detected in these fractions (Fig. 2). These fractions were dried in vacuo and applied to Infrared (IR) spectrometer to get some information on the structure of the lipid moiety of LP44. In the spectrometry, Pam₃-cysteine was used as a standard. The IR spectra of the lipid moiety of LP44 and Pam₃-cysteine exhibited signals around 2,900 cm⁻¹ and 1,700 cm⁻¹ which show the presence of fatty acid alkyl chains and typical ester bonds, respectively (Fig. 3 A, B). Lipid is bound to SH group of the N-terminal cysteine in LP from many microbes. The structure of N-terminal lipid of murine LP of E. coli is determined to be S-(2,3-bisalicyloyxpropyl)-N-palmitoyl-Cys-Ser-Ser-Asn-Lys-Ile-Asp-Glu-Leu-Ser-Ser-Asp⁻¹. Furthermore, Mühlradt, et al.23,44 identified a lipopeptide called as MALP-2, S-(2,3-bisalicyloyxpropyl)-Cys-Gly-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Lys, from M. fermentans which is capable of activating macrophages/microbes.

On the basis of these findings, the structure of the lipid moiety of LP44 is speculated to be S-(2,3-bisalicyloyxpropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe-Thr-Glu-Trp-Val-Ala⁻¹. In order to confirm whether a lipopeptide with the speculated
structure possesses the activity to activate GFh, S-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (FSL-1) was synthesized (Fig. 4). IR spectrum of FSL-1 (Fig. 3 C) also exhibited signals around 2,900 cm⁻¹ and 1,700 cm⁻¹ due to fatty acid alkyl chains and the ester bond as those of the HPLC-purified lipid moiety of LP44 and Pam₃-cysteine. In addition, it was confirmed that FSL-1 activates of GFh to produce inflammatory cytokines and an adhesion molecule (Fig. 5), suggesting that the active entity of LP44 is the N-terminal lipopeptide moiety, the structure of which is very similar to FSL-1.

Relationship between Structures and Biological Activities of the Diacylated Lipopeptide FSL-1 and Its Recognition by Toll-like Receptors 2 and 6

Braun⁶ first isolated LP (murein LP) from the cell wall of E. coli and determined its structure. The murein LP has potent mitogenic activity toward B lymphocytes, the activity resides in the N-terminal lipopeptide moiety and the delipidated LP was not biologically active⁸⁻¹⁰.⁶³. A well-defined series of lipopeptide analogs has been synthesized on the basis of the structure of the N-terminal lipopeptide of murein LP, and the relationship between their structures and biological activities has been investigated¹¹. The lipopeptides carrying two to five amino acids exhibit strong stimulatory activity comparable to that of native murein LP¹¹. In contrast, the lipopeptides containing only one amino acid are only marginally active, suggesting that the presence of a dipeptide structure is necessary for the expression of full biological activity¹¹. Lipopeptides containing two ester-bonded palmitoyl residues exhibit more potent mitogenic activity toward murine splenocytes than did a lipopeptide containing one ester-bonded palmitoyl residue⁴⁴, Rhodopsuedomonas viridis lipopeptides containing two ester-bonded palmitoyl residues and a free N-terminus exhibit more potent activity toward murine splenocytes than do lipopeptides containing three palmitoyl residues or N-terminally elongated lipopeptides⁴⁵. Thus, many aspects of the relationship between the structures and biological activities of bacterial triacylated lipopeptides have been elucidat-
ed. Therefore, we focused on the relationships between the structures and biological activities of mycoplasmal diacylated lipopeptides. The \textit{M. fermentans}-derived lipopeptide MALP-2 has been shown to be a potent macrophage activator\textsuperscript{13,14}.

FSL-1 possessed the activity to induce the production of IL-6, IL-8 and monocyte chemoattractant protein-1 by GFh (Fig. 5). The activity increased with increase in the concentration up to 10 nM and then decreased (Fig. 5). An experiment was carried out to determine whether FSL-1 is capable of activating THP-1 cells, because MALP-2 which is similar to FSL-1 in framework structure (Fig. 4) is a potent macrophage–activating lipopeptide\textsuperscript{13,14}. FSL-1 activated the cells to produce TNF-\textalpha at concentrations ranging from 1 nM to 100 nM (Fig. 6). The level

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**Fig. 3** IR spectra of Pam\textsubscript{3}-cysteine (A), HPLC-purified lipid moiety of LP44 (B) and FSL-1 (C)

Arrows 1 and 2 are signals showing the presence of fatty acid alkyl chains and typical ester bonds, respectively.
of the activity of FSL-1 was higher than that of MALP-2 (Fig. 6), suggesting that the difference in the amino acid sequence of diacylated lipopeptides affects the activity.

In order to further clarify the roles of the diacylglycerol and peptide portions of FSL-1 in the TNF-α production-inducing activity to THP-1 cells, the diacylglyceryl Cys (PamCys) and the peptide (Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe) portions of FSL-1 (Fig. 4) were examined for the activity. It was found that neither PamCys nor the peptide showed the activity at concentrations up to 100 nM (Fig. 7), suggesting that the whole lipopeptide structure of FSL-1 is essential for expression of the activity. To determine more precisely minimal molecular requirements for the activity to THP-1 cells, PamCys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Arg (FSL-2) in which the hydrophobic phenylalanine residue at the C-terminus of the peptide portion of FSL-1 has been converted to a hydrophilic arginine residue, and S-(2,3-bis-stearyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe (FSL-3), in which palmitic acid (C16:0) has been converted to stearic acid (C18:0), were synthesized (Fig. 4). These lipopeptides were examined for the activity to induce TNF-α production by THP-1 cells. Three lipopeptides showed such activities but the levels of activity varied (Fig. 7).

That is, a single amino acid substitution from phenylalanine to arginine in the C terminus drastically reduced the activity, and a fatty acid substitution from palmitic acid to stearic acid also significantly reduced, although to a lesser extent, the activity (Fig. 7).

Takeuchi, et al. suggest that TLR2 requires TLR6 as a coreceptor for recognition of diacylated lipopeptides such as mycoplasmal lipopeptides. Therefore, experiments were carried out to determine the expression of TLR2 and TLR6 in THP-1 cells and GFh. RT-PCR analysis indicated that mRNAs of TLR2 and TLR6 were expressed in both types of cells and the expression levels were not upregulated by stimulation with FSL-1. The surface expression of TLR2 on THP-1 cells and GFh was also investigated by flow
Fig. 5 Production of IL-6, IL-8 and MCP-1 and surface expression ICAM-1 by HGF induced by FSL-1. HGF were cultured until confluency and then stimulated at 37°C for 15 h with various concentrations of FSL-1 in DME medium containing 0.1% human serum. The culture supernatants were collected and examined for amounts of IL-6, IL-8 and MCP-1, which were determined using EIA kits. The ICAM-1 expression was assessed by cell-EIA (50). Data, expressed as means ± SD from triplicate wells, are representative of three separate experiments.

cytometry. TLR2 was expressed in the cell surface of both THP-1 cells and GFh, but the expression level in the former is extremely higher than that in the latter.

In order to confirm whether FSL-1, FSL-2, FSL-3 and MALP-2 are recognized by TLR2 and TLR6, HEK293 cells were transiently transfected with TLR2 and/or TLR6 (hereafter, 293TLR2, 293TLR6 and 293TLR2/6 cells) together with a NF-κB luciferase reporter plasmid, and then examined for the NF-κB reporter activity after stimulation with these lipopeptides. The expression of TLR2 protein in HEK293 transfectants was confirmed by Western blotting, a confocal laser microscopy and flow cytometry (Fig. 8). FSL-1 stimulated the NF-κB reporter activity in 293TLR2 and 293TLR2/6, but not 293TLR6 cells, in a dose dependent manner (Fig. 9). In addition, the activity is higher in 293TLR2/6 cells than in 293TLR2 cells, supporting the finding of Takeuchi, et al. as described above. The finding that FSL-1 did not stimulate the activity in 293TLR6, suggesting that endogenous TLR2 is not functional. The reason why FSL-1 stimulated the activity in 293TLR2 cells not transfected with TLR6 may be explained by the idea that endogenous TLR6 functions as a coreceptor in 293TLR2 cells. Indeed, FSL-1 stimulated the NF-κB reporter activity in HEK293 cells transfected with a dominant negative gene of TLR6 with converting
proline residue at a position of 680 to histidine, but the level of activity was drastically reduced when compared with that in 293TLR2 (Fig. 10). As described above, the level of activity of FSL-1 to THP-1 cells was much higher than that of MALP-2, FSL-2 and FSL-3 (Figs. 4, 6). Therefore, experiments were carried out to reproduce these results in HEK293 transfected with TLR2 and/or TLR6. FSL-1, FSL-2, FSL-3 and MALP-2 showed the NF-κB reporter activity in 293TLR2/6 cells but the level of the activity decreased in this order (Fig. 11). The same result was obtained in the case of 293TLR2 cells, although the level of the NF-κB reporter activity was lower (data not shown). That is, substitutions of an amino acid at C terminus and a fatty acid and the difference in the amino acid sequence of the peptide portion affected the activity, suggesting that both diacyl and peptide portions are involved in the recognition by TLR2 and/or 6.

**Fig. 6** Production of TNF-α by THP-1 cells induced by FSL-1 and MALP-2. THP-1 cells were incubated at 37°C for 3 days in the presence of 100 nM vitamin D3 and then stimulated at 37°C for 15 h with various concentrations of FSL-1 (●) and MALP-2 (■) in RPMI 1640 medium containing 0.1% human serum. The culture supernatants were collected and examined for the amount of TNF-α using an EIA kit. Data, expressed as means±SD from triplicate wells, are representative of three separate experiments.

**Fig. 7** Production of TNF-α by THP-1 cells induced by FSL-1, PamCys, FSL-1 peptide and derivatives of FSL-1. THP-1 cells were incubated at 37°C for 3 days in the presence of 100 nM vitamin D3 and then stimulated at 37°C for 15 h with various concentrations of FSL-1 (●), PamCys (■), FSL-1 peptide (∆), FSL-2 (○) and FSL-3 (□) in RPMI 1640 medium containing 0.1% human serum. The culture supernatants were collected and examined for the amount of TNF-α using an EIA kit. Data, expressed as means±SD from triplicate wells, are representative of three separate experiments.

**Recognition of FSL-1 by TLR2**

1. **Design of TLR2 mutants**

*In vitro* studies with overexpression of TLR2 or TLR2-knockout mice have confirmed the essential role of TLR2 in signaling by LP and lipopeptides. It has recently been demonstrated that TLR2 requires TLR6 as a coreceptor for recognition of diacylated lipopeptides such as mycoplasmal lipopeptides. In addition, numerous lines of evidence have shown that several intracellular signaling pathways triggered by TLR stimulation lead to NF-κB activation. The cytoplasmic domains of TLR homologues to IL-1R have been demonstrated to be essential for signaling leading to NF-κB activation. Because each TLR is involved in signaling by different bacterial ligands, the extracellular domains of TLRs may define the specificities for various ligands. We therefore focused...
on the extracellular domain of TLR2 that is responsible for recognition of LP and lipopeptides, because it is not known how they are recognized by TLR2. Mitsuzawa, et al.\textsuperscript{38} have recently found by using several deletion mutants of TLR2 that the extracellular region Ser40-Ile64 of TLR2 is critical for the recognition of \textit{S. aureus} PGN. Furthermore, in order to answer the criticism that the conformational change due to the 25-amino acid deletion caused the receptor to be inactive, they confirmed that the synthetic peptide corresponding to the TLR2 region of Ser40-Ile64 competed with TLR2wt for PGN recognition.

On the basis of the report of Mitsuzawa, et al.\textsuperscript{38}, two types of TLR2 deletion mutants (TLR2\textsuperscript{115E} and TLR2\textsuperscript{112E}) as shown in Fig. 12 were used to determine the region of extracellular domain of TLR2 critical for recognition of the diacylated lipopeptide FSL-1. In addition, point mutants with a substitution of each of Glu178, Glu180 and Glu190 of TLR2 to alanine and a substitution of each of Leu115 and Leu132 to glutamic acid were made (Fig. 12). This is because the amino acid residues Glu178, Glu180 and Glu190 were suggested by the results of computer analysis to be surface-exposed and because Leu115 and Leu132 are located at a LRR of TLR2.

2. Expression of TLR2 and TLR2 mutants in transiently transfected HEK293 cells

For analysis of the recognition mechanism of FSL-1 by TLR2, HEK293 cells transiently transfected with TLR2, its mutants (Fig. 12) and TLR6 were used. The expression or cellular localization of proteins of wild type TLR2 (TLR2wt) and its mutants were confirmed by Western blotting and a laser scanning confocal microscopy, respectively (Fig. 8)\textsuperscript{49}. Proteins of TLR2wt and its mutants were found to be localized in the cell membrane, as indicated by colocalization with Con A used as an established marker for cell surface glycoproteins, and also in the cytosol, possibly the endoplas-
mic reticulum. That is, there were no big differences in localization patterns of between TLR2 mutants (TLR2^{L112E}, TLR2^{L115E}, TLR2^{E190A} and TLR2^{D540-164}) and TLR2^{wt}.

In order to evaluate surface expression of TLR2wt and its mutants, flow cytometric analysis was also carried out, because surface expression of TLR2wt and its mutants were responsible for the recognition of ligands. Proteins of TLR2^{L112E}, TLR2^{L115E}, TLR2^{E190A} and TLR2^{D540-164} as well as TLR2^{wt} were expressed on the cell surface of their transient transfectants (Fig. 8). Ratios of mean fluorescence intensities of samples stained with anti-TLR2 mAb to that of an isotype control were shown in each histogram of Fig. 8. The ratio of TLR2^{wt} was the highest of all tested, and the ratios of TLR2^{L115E}, TLR2^{L112E}, TLR2^{E190A} and TLR2^{D540-164} decreased in this order (Fig. 8).

![Fig. 9](image)

**Fig. 9** FSL-1-induced NF-κB activation in HEK293 cells transiently transfected with TLR2 and/or TLR6. HEK293 cells (1×10^5) were plated in 24-well plates and transfected transiently with TLR2^{wt}, TLR6^{wt}, and TLR2^{wt} and TLR2^{wt} (TLR2/6) together with an NF-κB reporter plasmid and Renilla luciferase control reporter plasmid. Cells were stimulated at 37°C for 6 h with 0.1 nM and 1.0 nM FSL-1. Results, expressed as the means±SD of triplicate wells, are representative of three separate experiments. See text for details.

![Fig. 10](image)

**Fig. 10** NF-κB activation in HEK293 cells transiently transfected with TLR2 and/or TLR6^{pass} by FSL-1. HEK293 cells (1×10^5) were plated in 24-well plates and transfected transiently with TLR2^{wt} and TLR2^{wt} and TLR6^{pass} (TLR2/6) together with an NF-κB reporter plasmid and Renilla luciferase control reporter plasmid. Cells were stimulated at 37°C for 6 h with 0.1 nM and 1.0 nM FSL-1. Results, expressed as the means±SD of triplicate wells, are representative of three separate experiments. See text for details.

![Fig. 11](image)

**Fig. 11** NF-κB activation in HEK293 cells transiently transfected with TLR2 and TLR6 by FSL-1, FSL-2, FSL-3 and MALP-2. HEK293 cells (1×10^5) were plated in 24-well plates and transfected transiently with TLR2^{wt} and TLR6 (TLR2/6) together with an NF-κB reporter plasmid and Renilla luciferase control reporter plasmid. Cells were stimulated at 37°C for 6 h with 0.1 nM and 1.0 nM FSL-1, FSL-2, FSL-3 and MALP-2. Results, expressed as the means±SD of triplicate wells, are representative of three separate experiments.
1. Deletion mutants

- ECD (1-589)
- ICD (630-784)

2. Point mutants in the region of M1-V200 of TLR2

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<th>ICD (630-784)</th>
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Fig. 12 Schematic representation of deletion and point mutants of human TLR2.

Symbols: ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain. Amino acid is written as a single-letter code in the amino acid sequence of Met1-Val200 of human TLR2.

3. NF-κB activation in HEK293 cells transiently transfected with TLR2 mutants

As described above, FSL-1 stimulated NF-κB reporter activity in HEK293 cells transfected with a TLR2 gene and in the cells transfected with both TLR2 and TLR6 genes, but not in the cells transfected with only a TLR6 gene, in a dose-dependent manner (Fig. 9). Therefore, the following experiments were carried out in HEK293 cells transiently transfected with TLR2wt or TLR2 mutant genes together with a TLR6 gene.

To try to determine which region of the N-terminal extracellular domain of TLR2 is responsible for FSL-1 signaling, we examined NF-κB reporter activity in HEK293 cells transiently transfected with each of two TLR2 deletion mutant genes together with a TLR6 gene. Two deletion mutants (TLR2<sup>Δ540-644</sup> and TLR2<sup>Δ530-539</sup>) were constructed, because the region Ser40-Ile64, but not Cys30-Ser39, of TLR2 was demonstrated to be essential for recognition of <i>S. aureus</i> PGN by TLR2<sup>Δ580</sup>. Although TLR2<sup>Δ540-644</sup> was expressed on the cell surface of the transfectant (Fig. 8), it failed to activate NF-κB when stimulated with FSL-1 (0.1, 1.0 and 10 nM) (Fig. 13). On the other hand, TLR2<sup>Δ530-539</sup> induced NF-κB reporter activity, but the level of activity was significantly reduced in comparison with that induced by TLR2wt (Fig. 13). The same results were obtained by HEK293 cells transiently transfected with each of TLR2 mutant genes, but not with a TLR6 gene, although the level of activity was lower.

These results suggest that TLR2<sup>Δ540-644</sup> and, to a lesser extent, TLR2<sup>Δ530-539</sup> are involved in the recognition of FSL-1 as well as <i>S. aureus</i> PGN.

Point mutants with a substitution of each of Glu178, Glu180 and Glu190 to alanine and a substitution of each of Leu115 and Leu132 located in a leucine-rich repeat (LRR) motif to glutamic acid were made (Fig. 14). TLR2<sup>E178A</sup>, TLR2<sup>E180A</sup>, TLR2<sup>E190A</sup> and TLR2<sup>L115E</sup> induced NF-κB activation, but TLR2<sup>L115E</sup> failed to induce NF-κB activation when stimulated with FSL-1 (Fig. 14A). In addition, TLR2<sup>L115E</sup> and TLR2<sup>E190A</sup> were expressed on the cell surface of the transfectants and the level of the expression of the former was...
slightly higher than that of the latter (Fig. 8). These results suggest that Leu115 is essential for recognition of FSL-1 by TLR2. Experiments were therefore carried out to determine whether Leu115 is involved in recognition of other TLR2 ligands, *M. salivarium* LP and *S. aureus* PGN. In the case of PGN stimulation, HEK293 cells were transiently transfected only with each of TLR2wt and mutant genes, but not with a TLR6 gene, because PGN is recognized by TLR2 in the absence of TLR6 (38, 43, 44). Neither LP nor PGN were recognized by TLR2 \( ^{1115E} \) in the same way as FSL-1 (Fig. 14 B, C), suggesting that Leu115 is a critical residue in recognition of these TLR2 ligands. A substitution of Glu190 to Ala reduced the level of NF-\( \kappa \)B activation in response to PGN stimulation but not to FSL-1 and LP (Fig. 14 A—C), suggesting that the recognition site for PGN is slightly different from that for LP or FSL-1.

On the basis of these results, we speculated that leucine residues located in the LRR motif around Leu115 are responsible for the recognition of FSL-1, and we made point mutants of TLR2 with a substitu-

tion of each of Leu107, Leu112 and Leu123 to glutamic acid. Expectedly, TLR2 \( ^{1107E} \), TLR2 \( ^{1112E} \) as well as TLR2 \( ^{1115E} \) failed to induce NF-\( \kappa \)B activation when stimulated with FSL-1 (Fig. 15 A). In addition, TLR2 \( ^{1112E} \) as well as TLR2 \( ^{1115E} \) was shown to be expressed on the cell surface of the transfectants (Fig. 8). However, TLR2 \( ^{1122E} \) induced NF-\( \kappa \)B activation, but the level of activation was significantly reduced in comparison with that induced by TLR2wt (Fig. 15 A). The same results were obtained when LP and PGN were used as stimulants (data not shown). Thus, it was found that Leu107, Leu112 and Leu115, and, to a lesser extent, Leu123 in the LRR motif are involved in the recognition of LP and PGN as well as FSL-1 by TLR2. This finding encouraged us to investigate whether substitutions of each of these leucine residues to a hydrophobic glycine, not to a hydrophilic glutamic acid, affect NF-\( \kappa \)B activation. Neither TLR2 \( ^{1125G} \) nor TLR2 \( ^{116G} \) induced the activation (Fig. 15 B). However, a substitution of TLR2 \( ^{1107G} \) induced the activation, but the level of activation was significantly reduced (Fig. 15 B).

On the basis of these results, it was considered that Leu107, Leu112 and Leu115 in an LRR motif of human TLR2 play important roles in the recognition of *M. salivarium* LP, *S. aureus* PGN and the diacylated lipopeptide FSL-1.

This is the first report to show that leucine residues in an LRR are critical for the recognition of PGN, diacylated LP and diacylated lipopeptide by TLR2, although Mitsuzawa, et al.\(^{38}\) have suggested on the basis of results of experiments using deletion mutants of TLR2 that an LRR around the region of Ser40-Ile64 is responsible for *S. aureus* PGN signaling. The structural characteristic of TLR proteins is that they possess LRR motifs, which appear to be involved in protein–protein interaction\(^{56,57}\). LRR motifs were first discovered in \( \alpha \)-glycoprotein of human serum\(^{58}\). LRRs are 20–29-residue sequence motifs present in a diverse group of molecules with differing functions\(^{54}\). Individual repeats correspond to structural \( \beta - \alpha \) units, consisting of a short \( \beta \)-strand and \( \alpha \)-helix approximately parallel to each other\(^{54}\). TLR2 possesses more than 10 LRRs. Alignment of the region around Leu107-Leu123 of TLR2 with an LRR consensus
Fig. 14  FSL-1-, LP-, and PGN-induced NF-κB activation in HEK293 cells transiently transfected with TLR2wt or point mutants together with TLR6. HEK293 cells (1×10⁵) were plated in 24-well plates and transfected transiently with either TLR2wt, TLR2 point mutants (TLR2E178A, TLR2E180A, TLR2E190A, TLR2L115E or TLR2L132E) together with TLR6, an NF-κB reporter plasmid and Renilla luciferase control reporter plasmid. Cells were stimulated at 37°C for 6 h with FSL-1 (0.1, 1.0 and 10.0 nM) (A), *M. salivarius* LP (0.1, 1.0 and 10.0 μg/ml) (B) or *S. aureus* PGN (10, 100, 1,000 ng/ml) (C). In the case of PGN, HEK293 cells were not transfected with TLR6wt. Results, expressed as the means±SD of triplicate wells, are representative of three separate experiments. See text for details.

sequence (LEXLXLCXCTXXXLXXL) described by Kobe and Deisenhofer suggests that Leu107 and Leu112 are located at a short β-strand and that Leu115 is located at an α-helix region (Fig. 16). Based on this speculation, a substitution of Leu107 and Leu112 located in the β-strand region to a hydrophilic amino acid, glutamic acid, would cause a small conformation change of the surfaced-exposed α-helix region, which in turn would abrogate the recognition by TLR2. This result suggests that each of these leucine residues is involved in recognition of diacylated LP and diacylated lipopeptide, and PGN.
Fig. 15  FSL-1-, LP- and PGN-induced NF-κB activation in HEK293 cells transiently transfected with TLR2wt or point mutants together with TLR6. HEK293 cells (1 × 10⁴) were plated in 24-well plates and transfected transiently with either TLR2wt, TLR2 point mutants (TLR2L107E, TLR2L115E, TLR2L118E or TLR2L123E) (A) or other TLR2 point mutants (TLR2L107G, TLR2L115E or TLR2L118G) (B) together with TLR6wt, an NF-κB reporter plasmid and Renilla luciferase control reporter plasmid. Cells were stimulated at 37°C for 6 h with FSL-1 (0.1, 1.0 and 10.0 nM). Results, expressed as the means ± SD of triplicate wells, are representative of three separate experiments. See text for details.

Fig. 16  Alignment of the region around Leu107-Leu123 of human TLR2 with an LRR consensus sequence (LEXLXXXCLTXXCLXXL) described by Kobe and Deisenhofer. Amino acid is written as a single-letter code.

Concluding Remarks

LP is a membrane-bound protein with a diacyl or triacyl group of the N-terminal lipid moiety by which it is anchored into the membrane and has been found extensively in Gram-positive and -negative bacteria, spirochetes such as Treponema pallidum and Borrelia burgdorferi, and Mycoplasma species. Many scientists have believed the monophylogenetic model for endotoxin is only LPS. However, Galanos, et al. have recently proposed that the mycoplasmal lipopeptide MALP-2 is an endotoxin because it possesses many classical endotoxin properties such as the cytokine production-inducing activity toward macrophages, mitogenic activities towards B lymphocytes, pyrogenicity, and lethal toxicity. In addition, Aliprantis, et al. have demonstrated that microbial LP possesses the activity to induce apoptosis and necrosis in various types of cells. Taken together, it is very likely that microbial LP play important etiological roles in diseases caused by these microbes. Especially, LP of wall-less microbes like mycoplasmas might play more important roles, because they interact directly with host cells. M. salivarium, a member of oral microbial flora, is suspected to play some etiological role in some cases of oral infections including periodontal diseases, but its etiological roles remain unknown. The present finding that the organism possesses membrane-bound LP capable of activating monocytes/macrophages and GFh may give a clue to clarify etiological roles in oral infections, especially periodontal disease. LPS, LTA and PGN of periodontopathic bacteria have mainly been studied as etiological agents in periodontal diseases, but their LP should be taken into account as an etiological agent. This is because periodontopathic bacteria possess LP in cell wall, which is capable of activating GFh and
macrophages and inducing cell death.

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