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Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by Toll-like receptors 2 and 6

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Running title: Recognition of mycoplasmal lipopeptides by TLR2 and 6

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Key words: Mycoplasma, diacylated lipopeptide, Toll-like receptor
Abstract
The lipopeptide FSL-1 [S-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam2CGDPKHPKSF] synthesized on the basis of the N-terminal structure of a Mycoplasma salivarium lipoprotein capable of activating normal human gingival fibroblasts to induce the cell surface expression of ICAM-1 revealed the activity to induce production of monocyte chemoattractant protein-1, IL-6 and IL-8. FSL-1 also activated macrophages to produce TNF-α as the M. fermentans-derived lipopeptide MALP-2 (Pam2CGNNDESNSIFKEK), a potent macrophage-activating lipopeptide did. The level of the activity of FSL-1 was higher than that of MALP-2. This result suggests that the difference in the amino acid sequence of the peptide portion affects the activity, because the framework structure other than the amino acid sequence of the former is the same as that of the latter. To determine minimal structural requirements for the activity of FSL-1, the diacylglyceryl Cys and the peptideportions were examined for the activity. Both portions did not reveal the activity. A single amino acid substitution from Phe to Arg and a fatty acid substitution from palmitic acid to stearic acid drastically reduced the activity. The similar results were obtained by the NF-κB reporter activity of FSL-1 to human embryonic kidney (HEK) 293 cells transfected with Toll-like receptor 2 and 6 together with a NF-κB dependent luciferase reporter plasmid.

These results suggest that both the diacylglyceroyl and peptide portions of FSL-1 are indispensable for the expression of biological activities and for the recognition by Toll-like receptors 2 and 6, and that the recognition of FSL-1 by Toll-like receptors 2 and 6 appears to be hydrophobic.

INTRODUCTION
Various bacterial cell wall components such as lipopolysaccharides (LPS), lipoteichoic acids (LTA), peptidoglycans (PGN) and lipoproteins (LP) have been shown to activate macrophages, fibroblasts or lymphocytes to induce production of cytokines (16). Escherichia coli LP were first characterized and sequenced by Braun et al. (9), and they have been demonstrated to be biologically active (5-8,20). The part of LP responsible for biological activities is demonstrated to be the N-terminal lipopeptide moiety, the structure of which is S-(2,3-bispalmitoyloxypropyl)-N-palmitoyl-Cys-Ser-Ser-Asp-Ala- (Pam3CSNNA-) (7).

Mycoplasmas, wall-less microorganisms, also possess LP capable of activating macrophages or fibroblasts (11, 27, 28, 31, 32). Mühlradt et al. (27, 28) have recently 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-bispalmitoyloxypropyl)Cys-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Lys (Pam2CGNNDESNSIFKEK). We have also found that Mycoplasma salivarium LP activate normal human gingival fibroblasts (GFh) to induce production of inflammatory cytokines and surface expression of ICAM-1, and have purified a 44-kDa LP (LP44) responsible for the activity (32). The structure of the N-terminal lipopeptide moiety of LP44 has been determined to be Pam3CGDPKHPKSFIGNWV- (32). The lipopeptide Pam3CGDPKHPKSF (FSL-1) synthesized on the basis of the N-terminal structure of LP44 showed the same activity as LP44 (32). The framework structure of FSL-1 is the same as that of MALP-2, but they differ in the amino acid sequence and length of the peptide portion. Mycoplasmal lipopeptides such as FSL-1 and MALP-2 contain two ester-linked fatty acids bound to glyceryl Cys and a free N-terminus of the peptide portion. It is of great interest to know structural requirements of these lipopeptides for expression of their biological activities.

A key element in the initiation of an innate immune response against pathogens is the recognition of components commonly found on the pathogen that are not normally found in the host. These components have been referred to as pathogen-associated molecular patterns (PAMPs) (24). Toll-like receptors (TLRs) have recently been identified and implicated as receptors for PAMPs such as LPS, PGN, LP (1, 2, 19, 23, 39). It has already been demonstrated that TLR2 functions as a receptor for microbial LP and lipopeptides (2-4, 10, 13, 17, 18, 22, 23, 36, 38) and that signaling by MALP-2 is mediated by TLR2 (10, 17, 18, 38). More recently, it has been demonstrated that TLR2
requires TLR6 as a coreceptor for recognition of diacylated lipopeptides (38).

In this study, therefore, experiments were carried out to further clarify the structure-function relationship of FSL-1 and the structural requirements of diacylated lipopeptides for recognition by the receptor consisting of TLR2 and TLR6.

MATERIALS AND METHODS

Antibodies, Reagents and Cells. DMEM, RPMI 1640 medium, penicillin G, streptomycin and trypsin-EDTA were obtained from Gibco BRL (Rockville, MD). FITC- and peroxidase-conjugated goat anti-mouse IgG and FITC-conjugated anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

GFh prepared and used in our previous study (11) were cultured in DMEM containing 10% (vol/vol) FBS (Gibco), penicillin G (100 units/ml) and streptomycin (100 μg/ml) in plastic culture dishes. In this study, GFh between passages 6 and 8 were used.

A human acute monocytic leukemia cell line, THP-1 (40), was obtained from Health Science Research Resources Bank (Osaka, Japan). These cell lines were grown at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, penicillin G (100 units/ml) and streptomycin (100 μg/ml).

Human embryonic kidney (HEK) 293 cells obtained from ATCC (CRL-1573) were maintained in DMEM containing 10% FBS, penicillin G (100 units/ml) and streptomycin (100 μg/ml).

Synthesis of lipopeptides. FSL-1 and its derivatives (FSL-2, FSL-3) and MALP-2, the structures of which are listed in Fig. 1, were synthesized as follows. The side chain-protected GDPKHSPKSF, GDPKHSPKSR or GNNDESNISFKFK was built up with an automated peptide synthesizer, model 433 (Applied Biosystems, Foster City, CA). Fmoc-S-(2,3-bispalmitoyloxypropyl)-cysteine and Fmoc-S-(2,3-bisstearyloxypropyl)-cysteine (Novabiochem, Laefelfingen, Switzerland) were manually coupled to the peptide-resin by using a solvent system of 1-hydroxy-7-azabenzotriazole-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/CH2Cl2-DMF. The Fmoc and resin were removed from the lipopeptide by trifluoroacetic acid. The lipopeptides were purified by preparative HPLC with a reverse-phase C18 column (30 x 250 mm). The purities of these lipopeptides were confirmed by analytical HPLC with a reverse-phase C18 column (4.6 x 150 mm) to be more than 90%. These lipopeptides were used without separation of S, R stereoisomer. These lipopeptides were originally dissolved in PBS containing 10 mM n-octyl-β-glucopyranoside (OG) in PBS and diluted with PBS to reduce the OG concentration to less than 0.5 mM when used for stimulation.

Cytokine production. GFh (10^4 cells) were seeded in a 96-well flat-bottomed microplate. After the GFh had reached confluency, the cells were stimulated at 37 °C for 15 h with lipopeptides in a humidified atmosphere of 5% CO2 in DMEM supplemented with 0.1% (vol/vol) human serum. The culture supernatant was collected and examined for production of IL-6 and IL-8 by using an HU IL-6 cytoset (Biosource International Inc., Camarillo, CA). Monocyte chemoattractant protein-1 (MCP-1) was measured by using a MCP-1 Immunoassay (R&D Systems, Minneapolis, MN). THP-1 cells were incubated at 37 °C for 3 days in the presence of 100 nM vitamin D3. After a 0.2-ml volume of cell suspension of THP-1 (5 x 10^5 cells) in each well of a 96-well tissue culture plate had been incubated at 37 °C for 15 h with FSL-1 in the culture medium supplemented with 0.1% (vol/vol) human serum, the culture supernatant was collected by centrifugation at 400 x g for 10 min. TNF-α in the supernatant was determined by using an HU TNF-α cytoset (Biosource).

Cloning of Human TLR2, TLR6 and dominant-negative TLR6. The cDNAs of human TLR2 and 6 were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of RNA isolated from THP-1 cells. The cDNAs of TLR2 and TLR6 were cloned into a pEF6/V5-His TOPO vector (Invitrogen Co., Carlsbad, CA) and the constructs were referred to as TLR2- and TLR6-TOPO,
respectively. The DNA sequences were confirmed by a dideoxy chain termination method of Sanger using ABI-PRISM 3100 Genetic Analyzer (Foster City, CA). The dominant-negative gene of TLR6 with a substitution of Pro residue at a position of 680 to His (TLR6\(^{Pro680His}\)) was produced by a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions with a TLR6-TOPO construct.

**NF-κB Reporter Assay.** Activation of NF-κB was measured as described previously (26). Briefly, HEK293 cells were plated at 1 x 10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) with 30 ng of an NF-κB luciferase reporter plasmid (pNF-κB-Luc, Stratagene) and 3.5 ng of a construct-directing expression of Renilla luciferase under the control of the constitutively active thymidine kinase promoter (pRL-TK, Promega Co., Madison, WI), together with 166.5 ng of each transfectant gene of TLR2, TLR6 and TLR6\(^{Pro680His}\).

Twenty-four hours after transfection, the cells were stimulated for 6 h with FSL-1, FSL-2, FSL-3 or MALP-2 in the absence of FBS and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

**Generation of polyclonal antibody to human TLR2.** An extracellular domain of TLR2 protein (Met\(^1\)-Arg\(^{508}\)) was subcloned into the pGEX-2T (Amersham Pharmacia Biotech, Tokyo, Japan) vector by the PCR method to generate a GST-TLR2 fusion protein and transformed into *E. coli* DH5α. The fusion protein formed inclusion bodies in the cells. Therefore, they were collected from the cells lysed in 20 mM Tris-HCl (pH 8.0) containing 0.5% NP-40 after treatment with lysozyme by centrifugation at 8,000 x g for 30 min. The purified inclusion bodies were subjected to SDS-PAGE, and the band corresponding to the GST-TLR2 fusion protein was excised from the SDS-PAGE gels and then homogenized. The homogenate was suspended in PBS and used for immunogen after being mixed at a ratio of 1:1 with Freund complete adjuvant. The polyclonal anti-TLR2 Ab was generated by immunizing Japanese White rabbits with the mixture. On days 1, 14 and 28, rabbits were injected into five subcutaneous and intramuscular sites. Sera were drawn 1 week after the final immunization and used as polyclonal anti-TLR2 Ab.

**Confocal Laser Microscopic Analysis of the Expression of TLR2 on the Cell Surface of HEK293 cells.** HEK293 cells were grown on poly-L-lysine-coated coverglasses on the day before transfection. The cells were transiently transfected by Fugene 6 transfection reagent with a TLR2 gene. After a 24-h incubation, the medium was removed and the cells were incubated with serum-free DMEM containing rhodamine-conjugated Con A (5 μg/ml) (Molecular Probes, Eugene, OR), followed by methanol fixation (4 min at -20 °C). The cells were washed with PBS and then 1 μg/ml rabbit polyclonal anti-TLR2 Ab generated in our laboratory diluted with PBS and further incubated with Alexa-anti-rabbit IgG Ab (Molecular Probe) at room temperature for 45 min. The cells were finally washed three times with PBS, sealed in the presence of 90% glycerol, and observed using a laser microscope (LSM510, Carl Zeiss, Tokyo, Japan). Digital images were acquired and processed using Adobe Photoshop, version 5.0 (Mountain View, CA).

**Surface expression of TLR2 in THP-1 cells by flow cytometry and the expression of mRNAs of TLR2 and TLR6 by RT-PCR.** In order to assess the surface expression of TLR2 on THP-1 cells by flow cytometry, a single cell suspensions of THP-1 (10^6) cells were incubated at 4 °C for 1 h with or without anti-TLR2 (TL2.1) and then with FITC-conjugated anti-mouse IgG. The surface expression was measured using the flow cytometer EPICS (Beckman Coulter). Anti-TLR2 mAb (TL2.1) was generated as described previously (12).

The RNAs were prepared from THP-1 cells by using an RNeasy kit (QIAGEN Inc., Chatsworth, CA) according to manufacturer's instructions and was dissolved in 50 μl of RNase-free water. By using a RT-PCR kit (Takara Shuzo Co., Ltd., Shiga, Japan), the RNAs (0.1 μg) were transcribed with AMV reverse transcriptase by using the anti-sense primer of β-actin (21) and those of human TLR2 and TLR6 (35). The specificities of the primers for TLR2 and TLR6 were confirmed by Southern hybridization with a probe coding the internal sequence. The RT reaction was performed in an automated DNA thermal cycler according to manufacturer's instructions. Briefly, a 1-μl
volume (0.1 μg) of RNA was adjusted to a total volume of 20 μl in 10 mM Tris-hydrochloride (pH 8.3) containing 1 μM each of dATP, dCTP, dTTP and dGTP; 1 μM of the antisense primer for the cytokine; 5 mM MgCl₂; 50 mM KCl; 20 U of an RNase inhibitor; and 5 U of AMV reverse transcriptase. The RNA was transcribed at 55 °C for 30 min after incubation at 30 °C for 10 min and was then denatured at 99 °C for 5 min and cooled at 5 °C for 5 min. The resulting mixture containing cDNA was added to 80 μl of a mixture containing 10 mM Tris-hydrochloride (pH 8.3), 0.25 μM of each of the sense primer for each of β-actin and TLR2, 1.8 mM MgCl₂ and 1 U of Taq polymerase (Takara) and was amplified by PCR as follows: after 2 min of denaturation at 94 °C, 28 amplification cycles (30 sec of denaturation at 94 °C, 30 sec of annealing at 60 °C and 1.5 min of extension at 72 °C) were performed. The PCR products were separated on 2% gel of NuSieve 3:1 agarose (FMC, Rockland, ME) in 0.5 x TBE buffer containing ethidium bromide (5 μg/ml).

**Western blotting.** The transfected HEK293 cells grown in a 6-well plate were washed twice with ice-cold PBS and then lysed by 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol and 50 mM DTT (an SDS sample buffer) in the presence of inhibitor cocktails of proteases (Sigma) and boiled for 10 min. The lysates were centrifuged at 14,000 rpm for 10 min, and the resulting supernatants containing cytosolic and membrane proteins were collected. Proteins in the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated at 4 °C overnight with polyclonal anti-TLR2 Ab as described above and then with peroxidase-conjugated anti-rabbit IgG Ab. Immunoreactive proteins were detected by using ECL detection reagents (Amersham Pharmacia).

**RESULTS**

**Activation of HGF by FSL-1.** It has been demonstrated that FSL-1 synthesized on the basis of the N-terminal structure of LP44 is capable of activating HGF to induce the cell surface expression of ICAM-1 (32). First, experiments were carried out to determine whether FSL-1 induced the production of IL-6 and IL-8 by HGF, because *M. salivarium* LP, including LP44, can induce the production of these cytokines by HGF (32, 33). FSL-1 possessed the activity to induce the production of IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) by HGF (Fig. 2). The activity increased with increase in the concentration up to 10 nM and then decreased (Fig. 2). Thus, in addition to previous findings (32), this finding also suggests that FSL-1 is the N-terminal lipopeptide moiety of LP44 and is its active site.

**Activation of a monocytic cell line, THP-1 cells, by FSL-1.** 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. 1) is a potent macrophage-activating lipopeptide (27, 28). FSL-1 also activated the cells to produce TNF-α at concentrations ranging from 1 nM to 100 nM (Fig. 3). The level of the activity of FSL-1 was higher than that of MALP-2 (Fig. 3), suggesting that the difference in the amino acid sequence of diacylated lipopeptides affects the activity.

**Structure-function relationship of FSL-1.** In order to further clarify the roles of the diacylglycerol and peptide portions of FSL-1 in its TNF-α production–inducing activity to THP-1 cells, the diacylglycerol Cys (Pam₂Cys) and the peptide (CGDPKHSPKSF) portions of FSL-1 (Fig. 1) were examined for the activity. It was found that neither Pam₂Cys nor the peptide showed the activity at concentrations up to 100 nM (Fig. 4), suggesting that the 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, Pam₃CGDPKHSPKSR (FSL-2) in which the hydrophobic Phe residue at the C-terminus of the peptide portion of FSL-1 has been converted to a hydrophilic Arg residue, and S-(2,3-bisstearyloxypropyl)-CGDPKHSPKSF (FSL-3), in which palmitic acid (C16:0) has been converted to stearic acid (C18:0), were synthesized (Fig. 1). 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. 4). That is, a single amino acid substitution from Phe to Arg 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. 4).

**Expression of mRNAs of TLR2 and TLR6 in THP-1 cells and HGF.** Takeuchi et al. (38) 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 HGF. 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 (Fig. 5). The surface expression of TLR2 on THP-1 cells and HGF was also investigated by flow cytometry. TLR2 was expressed in the cell surface of both THP-1 cells and HGF, but the expression level in the former is extremely higher than that in the latter (Fig. 5).

**Recognition of mycoplasmal lipopeptides by TLR2 and/or TLR6.** 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. First, we attempted to define the cellular localization of the TLR2 protein in transiently transfected HEK293 cells by a laser scanning confocal microscope (Fig. 6). TLR2 protein was detected in the transfectant and was localized in the cell membrane indicated by colocalization with concanavalin A used as an established marker for cell surface glycoproteins and also in the cytosol (Fig. 6A and B).

FSL-1 stimulated the NF-κB reporter activity in 293TLR2 and 293TLR2/6, but not 293TLR6 cells, in a dose dependent manner (13). In addition, the activity is higher in 293TLR2/6 cells than in 293TLR2 cells, supporting the finding of Takeuchi et al. (38) 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 Pro residue at a position of 680 to His, but the level of activity was drastically reduced when compared with that in 293TLR2 (Fig. 7).

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 (Fig. 3 and 4). 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. 8). 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 the 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.

**DISCUSSION**

Multicellular organisms have developed various defense mechanisms that have the capacity to protect the host by destroying invading microbes and neutralizing their virulence factors. The immediate response to microbial pathogens, which is coordinated by the innate immune system, is characterized by the de novo production of mediators that either kill the pathogens directly or induce phagocytic cells to ingest and kill them. LPS, a glycolipid of the outer cell membrane of Gram-negative bacteria, is one of the most potent known stimulators of immune responses, including cytokine production by macrophages/monocytes (16). Bacterial LP have been shown to possess LPS-like biologic activities (16). Although LP and LPS are recognized by TLR2 and TLR4, respectively (1-4, 10, 17, 19, 21-23, 36-39), they share many characteristics, including a biologically active lipid modification, cell types that are responsive, and types of responses that are included. Braun et al. (9) 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 active (5-8, 20). 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 investigated (5). The lipopeptides carrying two to five amino acids exhibit strong stimulatory activity comparable to that of native murein LP (5). 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 activity (5). Lipopeptides containing two ester-bonded palmitoyl residues exhibit more potent mitogenic activity toward murine splenocytes than did a lipopeptide containing one ester-bonded palmitoyl residue (34). 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 lipopeptides (25). Thus, many aspects of the relationship between the structures and biological activities of bacterial triacylated lipopeptides have been elucidated.

In this study, we focused on the relationships between the structures and biological activities of mycoplasmal diacylated lipopeptides. The M. fermentans-derived lipopeptide MALP-2 has been shown to be a potent macrophage activator (27, 28). We also found that the lipopeptide FSL-1 synthesized on the basis of the structure of the N-terminal lipopeptide moiety of M. salivarium LP44 (32) is able to activate HGF to induce the cell surface expression of ICAM-1. In addition, this study demonstrated that FSL-1 possesses activities to induce production of inflammatory cytokines by macrophages as well as HGF. To determine the minimal molecular requirements for the activity of FSL-1 toward macrophages, Pam3Cys and the peptide portions were examined for their activities. It was found that both portions failed to activate macrophages, suggesting that the whole structure of the lipopeptide is required for the expression of the macrophage-stimulating activity. This speculation is supported by the results of a previous study showing that nonlipidated MALP-2 and Pam3Cys failed to activate monocytes/macrophages (15).

FSL-2, FSL-3 and MALP-2 were less active toward THP-1 cells and TLR-transfected HEK293 cells than was FSL-1 (Figs 4 and 8), suggesting that both the fatty acid of the diacylglycerol portion and the amino acid sequence of the peptide portion are indispensable for TLR2/6-mediated signaling. The finding that a single amino acid substitution from Phe (FSL-1) to Arg (FSL-2) reduced the activity also suggests that the hydrophobic interaction plays an important role in recognition of the peptide portion of mycoplasmal lipopeptides by the receptor. Furthermore, this is supported by the present finding that FSL-1 was more active toward macrophages and TLR2/6-transfected HEK293 cells than was MALP-2 (Figs 4 and 8) containing a hydrophilic Lys residue at the C-terminus (Fig.1). The finding that FSL-2 was more active toward HEK293 cells transfected with TLR2/6 than MALP-2 may suggest that the size as well as hydrophobicity of the peptide portion of the lipopeptide affect the recognition of the lipopeptide by TLR2/6. Furthermore, there is a possibility that the difference in the activities of these synthetic lipopeptides is attributed to the difference in ratios of R- and S-isomers of these lipopeptides, because the R-stereoisomer of MALP-2 is known to be more active to macrophages than the S-stereoisomer (36). Although the TNF-α production-inducing activity of FSL-2 to THP-1 cells was lower than that of FSL-3 (Fig. 4), the former was higher than the latter in the NF-κB reporter activity to HEK293 transfectants (Fig. 8). This discrepancy may be explained by the difference in the assay system. Namely, the TNF-α production-inducing activity was measured by using a monocytic cell line, THP-1 cells, whereas the NF-κB reporter activity was measured by using HEK293 cells transiently transfected with cloned human TLR2 and/or TLR6 genes. In addition, the sensitivity of the former assay system appears to be much lower than that of the latter, judging from the concentrations of FSL-1 used for these experiments (Figs. 4 and 8).

LP are membrane-bound proteins with a diacyl 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 burgdoferi, and Mycoplasma species. Many scientists have believed the monopoly that 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 endotoxic properties such as the cytokine production-inducing activity toward macrophages, mitogenic activities towards B lymphocytes, pyrogenicity, and lethal toxicity (14). However, we think that LP rather than lipopeptides is an important microbial endotoxin, because lipopeptide is only the N-terminal part of LP.

Studies are in progress to compare biological activities of LP with those of LPS in order to characterize the endotoxic and pathogenic properties of microbial LP.

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**FIGURE 1.** Structures of FSL-1, Pam2Cys, FSL-1 peptide and derivatives of FSL-1.
FIGURE 2. Production of IL-6, IL-8 and MCP-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. Data, expressed as means ± standard deviations from triplicate wells, are representative of three separate experiments.
**FIGURE 3.** 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 ± standard deviations from triplicate wells, are representative of three separate experiments.
FIGURE 4. Production of TNF-α by THP-1 cells induced by FSL-1, Pam2Cys, 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 (●), Pam2Cys (■), 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 ± standard deviations from triplicate wells, are representative of three separate experiments.
FIGURE 5. Expressions of mRNA of TLR2 and TLR6 and surface expression of TLR2 molecule in THP-1 cells and HGF. THP-1 cells and HGF were stimulated with 100 ng/ml of FSL-1 (S) or not stimulated (N). THP-1 cells incubated at 37 °C for 3 days in the presence of 100 nM vitamin D3 and HGF cultured until confluency were stimulated with 100 ng/ml of FSL-1 (S) or not stimulated (N). The expressions of mRNAs of TLR2 and TLR6 were assessed by RT-PCR. The cells were stained with mouse IgG2a (a dotted line) and anti-TLR2 (TL2.1) (a solid line) for flow cytometric analysis. See text for details.
FIGURE 6. Expression of the TLR2 molecule in HEK293 transient transfectants by Western blotting and confocal microscopic analysis. A. HEK293 cells were transiently transfected with or without (TOPO, an empty vector) a TLR2 gene and lysed in the SDS sample buffer. The lysates were centrifuged, and the resulting supernatants containing cytosolic and membrane proteins were collected. Proteins in the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated at 4 °C overnight with polyclonal anti-TLR2 Ab and then with peroxidase-conjugated anti-rabbit IgG Ab. B. HEK293 cells were transiently transfected with a TLR2 gene were incubated with rhodamine-conjugated concanavalin A. The cells were fixed and immunostained with a polyclonal rabbit anti-TLR2 Ab and then FITC-anti-rabbit IgG mAb. See text for details.
FIGURE 7. NF-κB activation in HEK293 cells transiently transfected with TLR2 and/or TLR6<sup>P680H</sup> by FSL-1. HEK293 cells (1 x 10<sup>5</sup>) were plated in 24-well plates and transfected transiently with TLR2<sup>wt</sup>-TOPO and TLR2<sup>wt</sup>-TOPO and TLR6<sup>P680H</sup>-TOPO (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. Statistical significance was analyzed by t test. Symbols: *, P<0.01; **, P<0.05.
FIGURE 8. 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 x 10^5) were plated in 24-well plates and transfected transiently with TLR2wt-TOPO and TLR6 -TOPO (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. See text for details. Statistical significance was analyzed by t test. Symbols: *, P<0.01; **, P<0.05.