A Mycoplasma fermentans-derived Synthetic Lipopeptide Induces AP-1 and NF-κB Activity and Cytokine Secretion in Macrophages via the Activation of Mitogen-activated Protein Kinase Pathways

(Received for publication, June 9, 1998, and in revised form, September 20, 1998)

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Mycoplasma lipoproteins have been demonstrated to stimulate mononuclear cells and induce proinflammatory cytokine secretion. In this paper, we show that a synthetic analog of the Mycoplasma fermentans membrane-associated lipopeptide macrophage-activating lipopeptide-2 (MALP-2) induces mRNA synthesis and protein secretion of interleukin-1β and tumor necrosis factor-α in human monocytes/macrophages and the murine macrophage cell line RAW 264.7, whereas the nonlipidated counterpart lacks this effect, underscoring the importance of protein acylation for cell activation. Synthetic MALP-2 (sMALP-2) induced the activation of MAPK family members extracellular signal regulated kinases 1 and 2, c-Jun NH2-terminal kinase, and p38 and induced NF-κB and AP-1 transactivation in macrophages. Whereas the specific p38 inhibitor SB203580 abrogated both cytokine synthesis and NF-κB and AP-1 transactivation in response to MALP-2, the selective MAPK/extracellular signal-regulated kinase-1 inhibitor PD-98059 decreased interleukin-1β and tumor necrosis factor-α production in response to sMALP-2 without affecting the transactivation of NF-κB or AP-1. These results indicate that activation of MAPKs by sMALP-2 is a crucial event leading to the expression of proinflammatory cytokines. Our findings demonstrate that the synthetic analog of MALP-2 reproduces the macrophage stimulation activity found in different fractions of mycoplasmas. Given that MALP-2 has been recently shown to be expressed at the surface of M. fermentans as a molecular entity, sMALP-2 constitutes a valuable surrogate for investigating immunomodulation by these microorganisms and evaluating the role that this activity plays in the development of inflammatory diseases associated with mycoplasma infections.

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‡This work was supported by the Pasteur Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1750 solely to indicate this fact.
¶The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; LAMP, lipid-associated membrane protein; MAPK, mitogen-activated protein kinase; MER, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MALP-2, macrophage-activating lipopeptide-2; sMALP-2, synthetic MALP-2; MOPS, 4-morpholinopropanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility gel shift assay; PMA, phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus; CHO, Chinese hamster ovary.
Macrophage Activation by M. fermentans Lipopeptide
diated by mycoplasmas and the role of this property in the patho-
genesis of these microorganisms.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—PCD-98059 and GST-c-Jun (1–79) were ob-
tained from Biomol Research Laboratories (Philadelphia, PA). SB
203580 was from Calbiochem (Nottingham, United Kingdom). Anti-
JNK1 (C17), anti-JNK2 (R23), anti-p38 (C20) polyclonal antibodies, 
A23178 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A380/
RK/Mpk2 assay kit was available through Upstate Biotechnology, Inc. 
(Łódź, Poland). The p42/44MAPK enzyme assay and [γ-32P]ATP (3000 
Ci/mmol) were commercially available from Amersham Pharmacia Bi-
tech (Les Ulis, France). Human and murine cytokine ELISAs were 
atained from R & D (Abingdon, UK) and Genzyme (Boston, MA), 
respectively. All cell culture media and reagents were obtained from 
Life Technologies (Cergy Pontoise, France). Consensus AP-1 and NF-κB oligonucleotides were purchased from Promega (Charbonnieres, France).

LPS, PMA, and A23178 were from Sigma (L’Isle d’Abeauquesnes, France).

Synthetic Lipopeptide—Peptide (CGNNDNSISFKEK) and acylated peptide corresponding to the previously published MALP-2 sequence (7) 
was kindly provided by Dr. Radolf. Acylated peptide was resuspended 
in cell lysates was determined by a micro-BCA assay (Pierce).

50 mM Na3VO4. For each 106 cells initially seeded, 100 ng/ml to stimulate THP-1 cells. For phosphotransferase assays, 
was extended for an additional 2 h. The mixtures were then centrifuged 
(7000 × g for 2 min at room temperature), and protein A-Sepharose 
beads were washed three times with buffer B (12.5 mM MOPS, pH 7.2, 
0.5 mM EGTA, 12.5 mM β-glycerol phosphate, 7.5 mM MgCl2, 1 mM dithiothreitol, 1% Nonidet P-40) containing 250 mM NaCl. The beads 
were resuspended in 10 μl of buffer B containing 10 mM MgCl2 and 1 
μM MnCl2 for phosphotransferase.

For p38 immunoprecipitation, the protocol was slightly modified. 10 μl of anti-p38 antibody was first coupled to A2G-Sepharose beads for 2 h 
at 4 °C and then washed with buffer B and added to 500 μl of cell 
lysates. Immunoprecipitation was allowed overnight at 4 °C with con-
tinuous rotation, and immunoprecipitates were analyzed as described above.

Measurement of Phosphotransferase Activity—ERK and p38 activation 
was determined in immunoprecipitates by means of measuring 
radioactively their respective phosphotransferase activities toward a 
peptide substrate using p42/44MAPK or p38/Mpk2 detection kits. Assays 
were performed according to the manufacturer’s instructions. Activity 
was expressed as [γ-32P]ATP cpm.

To measure JNK activation, 2 μg of GST-c-Jun was added to stress-
activated protein kinase/JNK immunoprecipitates in the presence 50 
μl of [γ-32P]ATP. The reactions were conducted at 30 °C for 30 min and then 
terminated by adding SDS sample buffer to 1× final concentration. 
Samples were analyzed by SDS-PAGE using 12% gels. Gels were 
fixed in 10% acetic acid and 50% methanol and then embedded in 
cellophane sheets, dried, and autoradiographed.

Nuclear Extract Preparation and Electrophoretic Mobility Shift As-
say—Nuclear extracts were prepared from 5 × 107 cells at indicated time 
intervals, nuclear extracts were prepared as described in Ref. 13, and protein 
content was determined by micro-BCA assay (Pierce). NF-κB and AP-1 
binding activity in nuclei of uninduced and induced cells was deter-
ned by an electrophoretic mobility gel shift assay (EMSA) as de-
scribed in Ref. 14 using 2–4 μg of nuclear proteins. EMSA gels were 
exposed to a PhosphorImager screen and quantitatively assessed by 
means of ImageQuant software (Molecular Dynamics). Supershift ex-
periments were performed by preincubating nuclear extracts with anti-
Rel family antibodies for 1 h at 4 °C before carrying out EMSA.

Plasmids, Cell Transfection, Activation, and Assay for Luciferase Activity 
The NF-κB, AP-1, and NF-AT (15, 16) luciferase reporter constructs were kindly provide by Dr. A. Acuto (Institut Pasteur, Paris, France).

RAW 264.7 cells were grown up to 80% confluence and then 
transfected with the indicated plasmids by the electroporation method 
method as described by Stacey et al. (17). CHO-CD14 and CHO-RSV cells were 
grown at 30 °C for 30 min and then 
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sMALP-2 Induces Transcription and Secretion of IL-1β and TNFα by Macrophages—M. fermentans sMALP-2 has been demonstrated to induce NO release from mouse macrophages (7). First, we have investigated the potential ability of sMALP-2 to stimulate cytokine production by monocytic cells. The murine macrophage cell line RAW 264.7 was challenged with increased concentration of sMALP-2, and TNFα production level was determined by ELISA. TNFα could be detected in RAW 264.7 supernatant when cells were stimulated with sMALP-2 at concentrations higher then 50 nM; the lowest doses did not stimulate TNFα production (Fig. 1A). In a 100–600 nM concentration range of sMALP-2, the TNFα level secreted by RAW 264.7 cells was found to be dose-dependent. A similar dose-response curve was obtained when measuring IL-1β production by murine macrophages under identical conditions (data not shown). In following experiments, sMALP-2 was used at 200 nM concentration; however, in some inhibition experiments the highest concentrations were applied.

sMALP-2 preincubation with polymyxin B (1000 units/ml) for 2 h before its addition to cell cultures had no effect on TNFα production, whereas polymyxin B completely abolished TNFα induction by LPS (data not shown). These data and the results of the Limulus amoebocyte lysate assay (see “Experimental procedures”) clearly indicate that the stimulatory effect of sMALP-2 preparations are not to be ascribed to endotoxin contamination.

As shown in Fig. 1, B and C, sMALP-2 at 200 nM induced the production of a considerable amount of IL-1β and TNFα by murine macrophage RAW 264.7 (Fig. 1B) and by human monocytes/macrophages as well (Fig. 1C). Similar results were obtained using the human myelomonocytic cell line THP-1 (data not shown). When human monocytes/macrophages or murine macrophages RAW 264.7 were challenged with LPS, TNFα, and IL-1β secretion levels were comparable with those obtained in response to sMALP-2 (Fig. 1, B and C). The nonlipidated form of MALP-2 failed to induce cytokine production by both human monocytes/macrophages and RAW 264.7 cells. In addition, tripalmitoyl S-glycerylcysteine was unable to stimulate cytokine production by these cells (Fig. 1, B and C). These findings demonstrate that lipid modification of MALP-2 peptide is required for macrophage stimulation and cytokine production.

We have further examined the levels of cytokine mRNA in RAW 264.7 cells stimulated with sMALP-2. Data from RNA slot hybridization, presented in Fig. 1D, clearly show that SMALP-2 stimulated IL-1β and TNFα mRNA synthesis, with a peak accumulation found at about 4 h after challenge.

Previous reports have strongly suggested that mycoplasma-derived membrane fractions stimulate monocytes/macrophages by a mechanism distinct from that of LPS and independent of CD14 and/or serum (1, 5, 20). We have therefore investigated whether sMALP-2 was able to induce NF-κB and AP-1 transactivation in CHO cells expressing human CD14 (11). CHO cells expressing CD14 (CHO-CD14) were transiently transfected with NF-κB or AP-1 luciferase reporter plasmid, and then cells were stimulated with LPS and sMALP-2. CHO cells harboring the empty expression vector (CHO-RSV) were transiently transfected with reporter plasmids and stimulated as described above. As expected, LPS induced an important luciferase activity (4–5-fold increases) in CHO-CD14 cells transfected with either NF-κB or AP-1 reporter plasmids, while no activation could be observed with CHO-RSV under similar conditions (Fig. 2). On the contrary, sMALP-2 induced neither NF-κB nor AP-1 transactivation in transfected CHO-CD14 cells (Fig. 2), even when it was used at higher concentrations (data not shown). Control experiments were performed by stim-
sMALP-2 does not activate NF-κB or AP-1 in the CHO-CD14 cell line. CHO cells expressing the human CD14 (CHO-CD14; left panel) or harboring the empty expression vector (CHO-RSV; right panel) were transiently transfected with either NF-κB/luciferase or AP-1/luciferase reporter plasmid. Transfected cells were either left untreated or treated with sMALP-2 (200 nM), LPS (100 ng/ml), or PMA (100 ng/ml). Luciferase activity was determined in cell lysates after 6-h incubation and normalized to protein content. Values from four distinct experiments ± S.D., each performed in duplicate, are expressed as the fold increase in luciferase activity seen following treatment relative to the luciferase activity measured in the absence of any stimulus.

sMALP-2 activates MAPK pathways and NF-κB and AP-1 transcription factors in macrophages—MAPKs are key molecules involved in macrophage activation by different bacterial products. We have therefore investigated the activation of the well characterized MAPKs, ERK1/2, p38, and JNK, in RAW 264.7 cells challenged with sMALP-2. Data presented in Fig. 3 clearly indicate that sMALP-2 significantly induced activation of all of the tested kinases. LPS has also been shown to induce activation of MAPKs in murine macrophages (21). MAPK activation kinetics in RAW 264.7 stimulated by LPS and sMALP-2 were compared. As shown in Fig. 3, LPS peak activation of ERK1/2, p38, and JNK was found at 15 min after stimulation, while peak activation of these kinases occurred at 30 min after challenge with sMALP-2. The kinetics of MAPK activation by sMALP-2 were comparable with those obtained when cells were stimulated with a crude extract of M. fermentans membrane lipoproteins (5). A similar pattern of MAPK activation was observed in human monocytes/macrophages and the THP-1 cell line stimulated with sMALP-2 (data not shown).

Transcription factors NF-κB and AP-1 are involved in the transcription of a variety of genes, including proinflammatory cytokines, during the immune response (22–24). Thus, we have addressed the ability of sMALP-2 to activate nuclear translocation of the transcription factors NF-κB and AP-1. Using 32P-labeled oligonucleotides containing consensus NF-κB or AP-1, we have performed EMSAs on nuclear extracts from RAW 264.7 cells stimulated with sMALP-2. As demonstrated in the Fig. 4, sMALP-2 was capable of inducing both NF-κB and AP-1 translocation. The specificity of AP-1 and NF-κB DNA binding were verified by competition analysis with an excess of nonradioabeled probes. NF-κB and AP-1 activation by sMALP-2 was found to be dose-dependent, and transcription factors were detectable starting from 100 nM of sMALP-2 concentration, confirming data presented in Fig. 1 (data not shown).

As depicted in Fig. 4A, similarly to the MAPK activation, LPS-mediated NF-κB translocation was more rapid (with a peak found at 2 h of stimulation) than that observed with sMALP-2 (peak at 4 h). The kinetics of NF-κB activation mediated by LPS reported herein were comparable with those previously described by Tebo et al. (25). We have further investigated by means of EMSA supershift the NF-κB isoforms induced by sMALP-2. The NF-κB p65 and p50 antibodies supershifted the protein complex, whereas c-Rel and RelB antibodies did not modify the pattern (data not shown), indicating that a p65/p50 heterodimer accounts for the NF-κB-translocated form.

sMALP-2 also activated AP-1 translocation in RAW 264.7 cells, and peak activity was observed at 4 h of stimulation. Although a basal AP-1 activation could be detected in these cells, PhosphorImager quantification of EMSA gels clearly shows that sMALP-2 induced a 4–5-fold increase in AP-1 DNA binding activity compared with control level (Fig. 4B). Once again, AP-1 translocation in response to LPS occurred earlier (peak at 2 h) than that induced by sMALP-2 (data not shown).

Pursuing the investigation of AP-1 and NF-κB induction by sMALP-2, we have studied the transactivation ability of these transcription factors.
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Factors by means of luciferase reporter plasmids. RAW 264.7 cells were transiently transfected with plasmids containing the AP-1- or NF-κB-dependent luciferase reporter gene and then stimulated with sMALP-2, and luciferase activity was determined in cell lysates. As demonstrated in Fig. 5, sMALP-2 induced a 3-fold increase in luciferase activity in cells transfected by either AP-1 or NF-κB reporter plasmid, indicating that the translocated factors can mediate transactivation. sMALP-2 failed to induce transactivation of the T cell-specific transcription factor NF-AT-dependent luciferase reporter gene. As a positive control, stimulation of NF-AT-transfected RAW 264.7 cells with PMA/A23187 (26) induced a 4-fold increase in specific luciferase activity (data not shown). Comparable levels of NF-κB- and AP-1-mediated luciferase induction were observed in LPS stimulation experiments (Fig. 5). sMALP-2 also induced AP-1- and NF-κB-dependent luciferase expression in THP-1 cells (data not shown).

**Involvement of MAPK Pathways in MALP-2-induced Cytokine Production and AP-1 and NF-κB Activation**—To examine the involvement of MAPK activation in sMALP-2-mediated cytokine production, we have used the specific inhibitors of MAPK pathways, SB203580 and PD-98059 (27, 28). At the highest used concentration, these two inhibitors, PD-98059 and SB203580, did not induce any cell toxicity as determined by microscopic observation and trypan blue uptake (data not shown).

SB203580 is a bicyclic imidazole compound able to specifically inhibit p38 (28). This inhibitor selectively blocked the sMALP-2-mediated activation of p38 in RAW 264.7 cells without significantly affecting the stimulation of ERK1/2 or JNK (data not shown). Preincubation of RAW 264.7 with SB203580 for 1 h prior to challenging with sMALP-2 blocked in a dose-dependent manner both IL-1β and TNFα production (Table I). Comparable inhibition effects of SB203580 were obtained when cells were stimulated with higher concentrations of sMALP-2 (data not shown).

PD-98059 is a synthetic compound that specifically inhibits the ERK-activating MAPK kinase MEK-1 (27, 29). This compound selectively inhibited the sMALP-2-mediated activation of ERK1/2 in murine macrophages without significantly affecting p38 or JNK stimulation (data not shown). The inhibition of MEK-1 by PD-98059 treatment partially inhibited, in a dose-dependent manner, cytokine production by RAW 265.7 in response to sMALP-2. Unlike SB203580, PD-98059 was unable to completely block sMALP-2 stimulation, and only 50% inhibition was obtained at 30 μM concentration (Table I) or at the highest doses (data not shown).
Macrophage Activation by M. fermentans Lipopptide

Table I
Effect of p38 and MEK-1 specific inhibitors on sMALP-2- and LPS-induced cytokine production

| Treatment | TNFα ng/ml | IL-1β pg/ml |
|-----------|------------|-------------|
| MeSO      |            |             |
| SB203580 (1 μM) | 3.92 ± 0.22 | 4.12 ± 0.36 |
| SB203580 (10 μM) | 3.11 ± 0.32 | 3.08 ± 0.19 |
| SB203580 (30 μM) | 1.59 ± 0.19 | 1.81 ± 0.28 |
| PD-98059 (1 μM) | 0.57 ± 0.18 | 0.68 ± 0.21 |
| PD-98059 (10 μM) | 3.78 ± 0.43 | 3.78 ± 0.25 |
| PD-98059 (30 μM) | 2.62 ± 0.24 | 3.2 ± 0.32 |
| UC        | 2.1 ± 0.2  | 1.95 ± 0.23 |
| sMALP-2   |            |             |
| LPS       | 35 ± 10    | 550 ± 62    |
| CTRL      | ND         | ND          |
| sMALP-2   | 50 (25.2%) | 75 (22.1%)  |
| LPS       | 29 (78%)   | 75 (36%)    |
| PD-98059  | 45 (53%)   | 461 ± 55.5% |
| sMALP-2   | 38 (84.5%) | 317 ± 49 (23.7%) |
| LPS       | 301 ± 46 (45.2%) | 253 ± 60 (48%) |

a UC, unstimulated cells.
b ND, not determined.

Fig. 6. Effect of p38 and MEK-1-specific inhibitors on sMALP-2-induced transcription factor activation in murine macrophages. RAW 264.7 cells were transiently transfected with either NF-κB- or AP-1-dependent luciferase reporter plasmids. Transfected cells were treated for 1 h with either SB203580 (p38 inhibitor) or PD-98059 (MEK-1 inhibitor) at the indicated concentrations and then stimulated with sMALP-2 (200 nM) for 6 h prior to cell lysis. MeSO (DMSO) (1%) was used as solvent control. Luciferase activity was assayed in stimulated and unstimulated cells (CTRL) and normalized to protein content. Values from three distinct experiments ± S.D., each performed in duplicate, are expressed as the fold increase in luciferase activity measured following stimulation relative to the luciferase activity observed in unstimulated cells.

Interestingly, treatment of RAW 264.7 cells with either PD-98059 or SB203580 yielded a similar inhibition pattern when LPS was used as stimulating agent (Table I). In addition, ERK1/2 and p38 pathway inhibitors, respectively, partially or completely inhibited cytokine production in the human cell line THP-1 stimulated with sMALP-2 or LPS (data not shown).

We have also assessed the effect of MAPK pathway inhibitors on sMALP-2-mediated AP-1 and NF-κB transactivation. RAW 264.7 cells were transiently transfected with either AP-1 or NF-κB reporter plasmids, and then cells were treated with PD-98059 or SB203580 prior to stimulation with sMALP-2. Whereas the MEK-1 inhibitor, PD-98059, at a concentration that inhibited 50% of cytokine production (see Table I) or higher, affected neither AP-1- nor NF-κB-dependent luciferase transactivation (Fig. 6), SB203580 significantly reduced both AP-1- and NF-κB-mediated transactivation (Fig. 6). The effect of SB203580 was dose-dependent and at 30 μM completely blocked AP-1 and NF-κB activities. Similar inhibitions were obtained when cells were stimulated with higher sMALP-2 concentrations (600 μM; data not shown). We have additionally assessed the effect of these inhibitors on LPS-induced NF-κB or AP-1 transactivation. In these experiments, inhibition profiles comparable with that reported with sMALP-2 were observed (data not shown). These data underscore the involvement of p38 pathway in the nuclear response to bacterial modulin in macrophages, while the ERK1/2 pathway appears to control distinct events.

DISCUSSION

M. fermentans is associated with arthritis in humans (30, 31) and it has been proposed as a putative co-factor in AIDS progression (30, 32). The ability of mycoplasmas to induce proinflammatory cytokines and to favor Th1 → Th2 switch might participate in the pathogenesis mechanisms of these bacteria (1, 2). Previous findings have clearly indicated that membrane lipoprotein fractions from M. fermentans are responsible of cytokine induction in macrophages. The precise characterization of the biochemical entities involved in such activation is an important step toward the understanding of the interaction of mycoplasmas and immune cells and necessary to delineate their contribution to the mycoplasma pathogenesis and clinical manifestations. MALP-2, a lipopeptide characterized from M. fermentans, has been shown to strongly induce NO synthesis by macrophages (7). Very recently, it has been reported that MALP-2 is a mycoplasma membrane-associated entity resulting from the processing of a precursor lipoprotein (33, 34). In the present study, we show that a synthetic analog of MALP-2 is capable of inducing the activation of NF-κB and AP-1 and the cytokine production by murine macrophages. We have also demonstrated the involvement of MAPK pathways in the signaling events triggered by MALP-2.

Our results confirmed and extended earlier findings concerning the macrophage activation by bacterial lipoproteins and lipopeptides. Lipoproteins from the outer membrane of Escherichia coli and its synthetically prepared NH2-terminal lipopeptide segments have been demonstrated to stimulate both human and murine macrophages and to induce the secretion of IL-1, TNFα, and IL-6 (35, 36). Spirochetal lipoproteins have also been shown to stimulate macrophages (37), and the lipid modification determines the ability to stimulate the production of cytokines by these cells (38). The importance of protein acylation to cell activation was further underscored by the fact that synthetic lipohexapeptides corresponding to the NH2 termini of the 47-kDa lipoprotein of Treponema pallidum and the acylated outer surface protein A (Ospa) of Borrelia burgdorferi also activated macrophages in terms of cytokine secretion, whereas the nonlipidated hexapeptides were without effect (39). In the present report, we also provided evidence showing that macrophage activation by MALP-2 is dependent upon acylation. Whereas both native MALP-2 and its synthetic an-
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a log have been previously shown to induce NO release from murine macrophages at a 1–100 ps range of concentrations (7), the effect of sMALP-2 on cytokine production by macrophages occurs at 100–600 nm concentration. For NO release assay, the simultaneous stimulation with IFN-γ and MALP-2 (7) could at least in part account for the significant high sensitivity of macrophages and the requirement of lower concentration of lipopeptide to induce NO activity with respect to cytokine production.

By using both electrophoretic mobility shift and transactivation assays, we have clearly shown that sMALP-2 stimulates the transcription factors NF-κB and AP-1. It has been previously reported that T. pallidum and B. burgdorferi lipoproteins and respective synthetic lipopeptides induce NF-κB translocation in different cell types (40, 41) with kinetics more rapid (maximal stimulation from 1 to 2 h) than that obtained with sMALP-2 (peak at 4 h). Interestingly, the kinetics of NF-κB and AP-1 induction by LPS were similar to that reported for the spirochetal products. It would be interesting to test whether the observed differences can be ascribed to the different lipopeptide structures.

Findings presented herein indicate that signaling pathways triggered in response to sMALP-2 and involved in cytokine production include ERK1/2 and p38 MAPKs. These same pathways with identical activation kinetics have been also implicated in macrophage response to M. fermentans membrane fractions (5). NF-κB and AP-1 triggering by sMALP-2 also involved MAPK pathways. In agreement with previously published reports indicating that p38 MAPK is required for the activation of several transcription factors, including cAMP response element-binding protein (42), c-Fos, and c-Jun (30–32, 43–45), we have shown that p38 pathway activation is required for AP-1 induction in response to sMALP-2. Interestingly, the p38 pathway was also found to be required for NF-κB response in sMALP-2-stimulated macrophages. Very recently, Bergmann et al. have shown that p38 inhibitor, SB203580, blocked NF-κB-mediated transactivation in response to TNFα without affecting NF-κB translocation (46). Accordingly, in our cells, SB203580 was unable to inhibit NF-κB translocation in response to sMALP-2 (data not shown). At present, how p38 MAPK may affect, directly or indirectly, NF-κB function is unclear, and the question remains open; however, our data underscore the important contribution of this pathway in nuclear response to bacterial lipopeptide stimuli. ERK1/2 has been clearly shown to contribute to the cell signaling resulting in cytokine production, but unlike the p38 MAPK pathway, it is not involved in sMALP-2-mediated NF-κB or AP-1 transactivation. Several other studies have shown that although the p42/44 MAP kinase pathway is activated in response to distinct stimuli including anisomycin, fibroblast growth factor, epidermal growth factor, 12-O-tetradecanoylphorbol-13-acetate, and UV irradiation, it is not involved in nuclear signaling (42, 44, 47). The signaling events controlled by ERK1/2 pathway remains to be clearly defined.

Our data demonstrate that the intracellular events leading to cytokine synthesis induced by both sMALP-2 or LPS are very similar, suggesting that common upstream pathways are triggered by these stimuli. In contrast to LPS, little is known about the mechanism by which bacterial lipoproteins and lipopeptide activate macrophages. The requirement for peptide or protein acylation strongly suggest that lipopeptides interact via the lipid moiety with currently unknown membrane molecule(s). Two recent papers have reported evidence suggesting that T. pallidum and B. burgdorferi lipoproteins and synthetic lipopeptides activate monocytes by a CD14-dependent pathway (48, 49). Actually, mouse anti-human CD14 antibodies were shown to block the activation of mononuclear cells by these spirochetal products (48) and to significantly decrease the sensitivity of endothelial cells to Borrelia lipoproteins (49). Very interestingly, Wooten et al. (49) have demonstrated that OspA and CD14 can form stable complexes. Yet, spirochetal lipoproteins and lipopeptides were shown to activate monocytes by a CD14-dependent pathway that fundamentally differs from that of LPS (48). We and others have previously demonstrated that M. fermentans lipoproteins induce monocyte activation by mechanisms distinct from that of LPS (1, 19). In this way, and in contrast to the results reported with spirochetal products, anti-CD14 antibodies have been shown to be inefficient in blocking the effects of mycoplasma lipoproteins on mononuclear cells (5, 20). In addition, human monocytic cell line THP-1 does not require vitamin D₃ treatment, which increases CD14 expression on cell membrane, to efficiently produce cytokine in response to mycoplasma lipoproteins.² Further experiments are necessary to evaluate whether mycoplasma and spirochetal derived lipoproteins and/or lipopeptides actually display some differences in triggering the monocyte activation.

As indicated above, it has been recently reported that MALP-2 derives from a precursor larger lipoprotein by post-translational processing (33, 34). Relative amount of MALP-2 and its precursor lipoprotein varied from one M. fermentans isolate to another. MALP-2 was initially characterized by selecting for M. fermentans isolates that are strong activators of macrophages (7). Interestingly, the selected strain II-29/1 abundantly expresses MALP-2 on the cell surface, whereas the precursor lipoprotein was undetectable (33, 34). To our knowledge, MALP-2 is the first membrane-associated lipopeptide so far described, and the nature of MALP-2 precursor lipoprotein processing is presently not understood. It remains to be determined whether the precursor lipoprotein displays an activity comparable with that of the lipopeptide. Preliminary data from our laboratory suggest that the recombinant precursor lipoprotein is capable of activating macrophages, but unlike MALP-2, acylation is not required. The confirmation of these results awaits the production of antibodies against different epitopes of the precursor lipoprotein and MALP-2. Given that MALP-2 is a surface-associated molecule able to activate macrophages, a synthetic analog of this lipopeptide constitutes a valuable tool to address the issue of the contribution of immunomodulation by mycoplasmas to the pathogenesis.

Acknowledgments—We thank Dr. I. Saint Girons for stimulating discussions and critical reading of the manuscript. We thank Dr. K. S. Wise for helpful discussions related to MALP-2 biology. We also thank Dr. J. D. Radolf (University of Texas, Southwestern Medical School, Dallas, TX) for providing acylated cysteine, Dr. R.J. Ulevitch (The Scripps Research Institute, La Jolla, CA) for providing the CHO-CD14 cell line, and Dr. O. Acuto (Institut Pasteur, Immunology Moleculaire, Paris, France) for the NF-AT, NF-κB, and AP-1 reporter plasmids. We acknowledge A. Henry-Dujeancourt and C. Prevost for technical assistance.

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*J. Biol. Chem.* 1998, 273:34391-34398.  
doi: 10.1074/jbc.273.51.34391

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