Involvement of Small GTPases in Mycoplasma fermentans Membrane Lipoproteins-mediated Activation of Macrophages*

(Received for publication, May 13, 1999, and in revised form, August 24, 1999)

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Mycoplasma fermentans lipoproteins (LAMPf) are capable of activating macrophages and inducing the secretion of proinflammatory cytokines. We have recently reported that mitogen-activated protein kinase (MAPK) pathways and NF-κB and activated protein 1 (AP-1) play a crucial role in the activation induced by this bacterial compound. To further elucidate the mechanisms by which LAMPf mediate the activation of macrophages, we assessed the effects of inhibiting small G proteins Rac, Cdc42, and Rho. The Rho-specific inhibitor C3 enzyme completely abolished the secretion of tumor necrosis factor α by macrophages stimulated with LAMPf and also inhibited the activation of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 kinase. In addition, we have shown that LAMPf stimulate Cdc42 and that inhibition of Cdc42 or Rac by dominant negative mutants abrogates LAMP-mediated activation of JNK and transactivation of NF-κB and AP-1 in the murine macrophage cell line RAW 264.7. These results indicate that small G proteins Rho, Cdc42, and Rac are involved in the cascade of events leading to the macrophage activation by mycoplasma lipoproteins.

A number of microbial cell wall products, including lipopolysaccharide, peptidoglycan fragments, lipoteichoic acid, and lipoproteins, have been demonstrated to activate macrophages (1). This activation result in the synthesis and secretion of a set of proinflammatory cytokines that can participate in the clinical manifestations associated with infectious diseases (2). Although mycoplasmas, the smallest self-replicating bacteria, are characterized by the absence of cell walls (3), they have been demonstrated to stimulate macrophages very efficiently (3, 4). Interestingly, some species of mycoplasmas, namely Mycoplasma fermentans, have been suspected of playing a role in rheumatoid arthritis (5). A number of studies show that mycoplasma-derived membrane lipoproteins (called LAMP for lipid-associated mycoplasma proteins) are the agents involved in the activation of macrophages leading to the secretion of proinflammatory cytokines (6, 7). It is therefore important to elucidate the mechanisms by which LAMP stimulate monocytes. We have recently demonstrated the role of the mitogen-activated protein kinase (MAPK) pathways in the cytokine secretion induced by M. fermentans-derived LAMP (LAMPf) (8). Challenging macrophages with LAMPf results in significant activation of MAPK family members p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun NH2-terminal kinase (JNK). Moreover, the selective inhibition of the different MAPK pathways dramatically affected the induction of cytokine secretion by LAMPf, underscoring the crucial role of these cascades in the LAMP-mediated effects. Furthermore, we have demonstrated that LAMPf are capable of inducing NF-κB, activated protein 1 (AP-1), and c-fos activation in macrophages and of stimulating NF-κB and AP-1 transactivation (9).

Certain small GTP-binding proteins control the activity of MAPKs. Rac1 and Cdc42 have been shown to regulate the activity of both JNK and p38 (10–12). In addition, Rho has been shown to control JNK activation in human epithelial cells (13). In this study we have examined whether small GTPases are involved in the activation of macrophages by LAMPf. Using different approaches to specifically inhibit Rac, Cdc42, and Rho in the murine macrophage cell line RAW 264.7, we have established their implication in the signaling cascades triggered by M. fermentans lipoproteins.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—GST-c-Jun (1–79) was obtained from Biomol Research Laboratories (Philadelphia). Anti-JNK1 (C17), anti-ERK (B23), anti-p38 (C20), anti-HA polyclonal antibodies, Cdc42 polyclonal antibody (SC-87), and A/G-Sepharose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The p38/RK/Mpk2 assay kit was commercially available from Amersham Pharmacia Biotech. TNFα enzyme-linked immunosorbent assay was obtained from Genzyme (Boston, MA). Chimeric C3 Iota (C3Ia)-toxin and the binding component of the Iota-toxin (Ib) were produced as described by Marvaudd and Popoff (14). Toxin B was produced from Clostridium difficile UP10463 as described by Just et al. (15).

Cell Culture, Stimulation, Plasmids, and Transfection Procedures—RAW 264.7 and THP-1 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. For stimulation experiments, cells were plated at a density of 10⁶ cells/ml and then cultivated overnight. For TNFα production, cells were stimulated with LAMPf (1 μg/ml) for 18 h. TNFα concentration was measured using a TNFα enzyme-linked immunosorbent assay according to the manufacturer’s instructions. Detection limit was 10 pg/ml. For phosphotransferase assays, cells were stimulated with LAMPf for 30 min as indicated above.
and then washed twice with ice-cold PBS containing 1 m NaCl, and lysed as described (8). Protein concentration in cell lysates was determined by micro-BCA assay (Pierce).

To assess the effect of bacterial toxins on LAMPf-mediated cell activation, cells were pretreated with either pertussis toxin (PTX) or toxin B at the indicated concentrations for 2 and 18 h, respectively, before stimulation with LAMPf. To address the effect of C3 enzyme, cells were preincubated with C3ia in the presence Ib (molar ration 1:1) at indicated concentrations for 1 h prior to LAMPf challenge. In control experiments, cells were pretreated with Ib alone as described above.

For transfection, RAW 264.7 cells were grown up to 80% confluence and then transfected with the indicated plasmids by the electroporation method as described by Stacey et al. (16). Briefly, cells were harvested at 5 × 10⁶ cells/ml in Dulbecco’s modified Eagle’s medium, and 200 μl (i.e. 10⁷ cells) were used for each electroporation. 10 μg of plasmid DNA were added to cells, which were then electroporated under the following conditions: 200 milliohms and 960 microfarads (time, 52 ms). Electroporated cells were left at room temperature for 10 min and then resuspended into 10 ml of culture medium and distributed in 24-well culture plates. Plasmids coding for Cdc42V12, Cdc42N17, RacV12, and Rac2N17 were provided by Dr. Afifi (CNRS, Saint Antoine, Paris). The HA-tagged JNK construct was provided by Dr. B. Dejerard (CNRS, Nice, France), and the HA-tagged ERK1 construct was obtained from Dr. J. Pierre (CNRS, Chatenay-Malabry, France). The NF-κB and AP-1 luciferase reporter constructs were kindly provided by Dr. A. Acuto (Institut Pasteur, Paris). Transfected cells were cultured in growth medium overnight and then either left unstimulated or stimulated with LAMPf or for 6 h. Cells were then harvested, protein concentration was determined by micro-BCA assay (Pierce), and luciferase activity was measured as described previously (17). Specific luciferase activity, determined in duplicate samples using an automated luminometer (La- mat LB 9501, EGG1G Berthold, Wilbad, Germany), was determined in arbitrary units after normalization to the protein content. Luciferase fold induction was calculated as the ratio of specific luciferase activity in the stimulated cells to the unstimulated ones.

Measurement of MAPK Activities—ERK, JNK, and p38 were immunoprecipitated with specific antibodies as described previously (8). ERK and JNK activation was determined by measuring radiolabeled phosphate incorporation into the respective phosphotransferase activities toward peptide substrate using p42/44 MAPK or p38/Mkp2 detection kits. To measure JNK activation, 2 μg of GST-c-Jun were added to SAPK/JNK immunoprecipitates in the presence 50 μM [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. Bands were visualized by autoradiography. The HA-tagged JNK construct was provided by Dr. B. Dejerard (CNRS, Chatenay-Malabry, France). The NF-κB and AP-1 luciferase reporter constructs were kindly provided by Dr. A. Acuto (Institut Pasteur, Paris). Transfected cells were cultured in growth medium overnight and then either left unstimulated or stimulated with LAMPf or for 6 h. Cells were then harvested, protein concentration was determined by micro-BCA assay (Pierce), and luciferase activity was measured as described previously (17). Specific luciferase activity, determined in duplicate samples using an automated luminometer (Lumat LB 9501, EGG1G Berthold, Wilbad, Germany), was determined in arbitrary units after normalization to the protein content. Luciferase fold induction was calculated as the ratio of specific luciferase activity in the stimulated cells to the unstimulated ones.

Affinity Precipitation of GTP-Cdc42 Using GST-CRIB—DNA for an extended Cdc42/Rac interactive binding (CRIB) domain from WASP (Wiskott-Aldrich syndrome protein) (amino acids 201–321) (18) was amplified by polymerase chain reaction using appropriate oligonucleotides, cloned into bacterial expression vector PGEX4T1 (Amersham Pharmacia Biotech), and expressed in bacterial expression vector PGEX4T1 (Amersham Pharmacia Biotech). The small GTPase proteins have been demonstrated to be involved in the activation of MAPK cascades in different cell models (21–23). To investigate the involvement of small GTPase proteins in macrophage activation by LAMPf, we decided to study the effects of selective inhibitors described for these proteins. Clostridium botulinum C3 enzyme specifically inhibits the small GTPase, Rho (24). Marvau and Popoff (14) have shown that Iota-toxin can internalize efficiently C3 enzyme (C3ia) into cells. The internalization is optimal in the presence Ib. We have tested the effect of C3ia treatment in the presence of Ib on the murine macrophage RAW 264.7 by evaluating its capacity to disrupt actin cytoskeleton. Treatment with C3ia/Ib (molar ratio 1:1) at 10⁻⁸ M for 1 h induced actin depolymerization in 90% of cells (data not shown). Under these conditions, C3 enzyme completely inhibited TNFα production in response to LAMPf by RAW 264.7 and THP-1 cells (Fig. 1), indicating that Rho is involved in the LAMPf-mediated macrophage activation. TNFα inhibition by C3ia/Ib was dose-dependent in the concentration range of 10⁻⁸ to 10⁻⁶ M (data not shown). As depicted in Fig. 1, Ib treatment alone did not modify the levels of TNFα in LAMPf-treated cells.

We have also examined the effect of C. difficile enterotoxin toxin B, which displays an inhibitory activity on the small GTPases Rac, Cdc42, and Rho (15). Treatment of cells with toxin B at 100 ng/ml prior to activation only slightly affected TNFα release by LAMPf-stimulated macrophages (Fig. 1). Concentration higher than 100 ng/ml did not exhibit stronger inhibition. In fact, in experiments where cells were treated with toxin B at 100 ng/ml or higher, only a small amount of the

![FIG. 1. Effect of bacterial toxins on LAMPf-mediated TNFα production by murine macrophage RAW 264.7 and human macrophages such as THP-1 cells. RAW 264.7 (light gray bars) and THP-1 (dark gray bars) cells were treated with PTX (100 ng/ml) for 2 h, toxin B (100 ng/ml) for 18 h, C3ia/Ib (10⁻⁶ M, molar ratio 1:1) for 2 h, or Ib (10⁻⁸ M) for 2 h. Cells were then stimulated with LAMPf (1 μg/ml), and TNFα was measured 18 h after stimulation. The results are the means of three separate experiments.](image)
Rac and Cdc42 Are Involved in LAMPf-induced MAPK Activation and NF-κB and AP-1 Transactivation—From the above experiments, the involvement of Rac1 and/or Cdc42 could not be clearly established, and we examined whether LAMPf was capable of activating Cdc42 in macrophage cells. We have used the CRIB domain from Wiskott-Aldrich syndrome protein that specifically interacts with GTP-bound Cdc42 (25) to determine the guanine nucleotide status of Cdc42 in macrophages challenged with LAMPf. RAW 264.7 cells were treated with LAMPf for different time intervals; the Cdc42 GTP-bound form was precipitated from cell lysates using a GST-CRIB construct and revealed by immunoblotting using anti-Cdc42 antibody. As shown in Fig. 3, a significant amount of GTP-bound Cdc42 could be detected in LAMPf-activated cells after 5 min of stimulation. These data clearly indicate that LAMPf affects the guanine nucleotide status of Cdc42 in RAW 264.7 cells.

To investigate the implication of these small GTPases in response to LAMPf, we used corresponding dominant negative forms. RAW 264.7 cells were transiently cotransfected with Rac dominant negative (RacN17) and with a plasmid expressing either HA-tagged JNK1 (HA-JNK) or HA-tagged ERK1 (HA-ERK). A similar set of cotransfection experiments was performed utilizing Cdc42 dominant negative (Cdc42N17). In both cases, cotransfected cells were stimulated with LAMPf for 30 min, and HA-JNK and HA-ERK were immunoprecipitated from corresponding cells using anti-HA antibody, and their activation was assessed. Control points included the use of constitutive active forms of Rac1 or Cdc42 (RacV12 and Cdc42V12, respectively). As depicted in Fig. 4, Rac or Cdc42 dominant negatives dramatically decreased the LAMPf-mediated JNK activation without significantly affecting LAMPf-mediated ERK activation. Interestingly, activation of Rac1 or Cdc42 by expression of the activated mutant forms (RacV12 or Cdc42V12) did not affect the activation of JNK by LAMPf, indicating that Rac1 and Cdc42 activation are necessary but not sufficient for LAMPf-induced JNK activation in macrophages. All of the Cdc42 and Rac constructs used herein were FLAG-tagged, and the expression of these constructs in transfected cells was verified by immunoblot using anti-FLAG antibody (data not shown).

We further investigated the effect of dominant negative Rac and Cdc42 on the activation of transcription factors AP-1 and NF-κB mediated by LAMPf. We therefore transiently cotransfected murine macrophage cell line RAW 264.7 with either RacN17 or Cdc42N17 and either NF-κB- or AP-1-luciferase reporter plasmids. Our data clearly show that RacN17 and Cdc42N17 were capable of abrogating both NF-κB- and AP-1-LAMPf-mediated activation (Fig. 5). Cotransfection with activated forms of Rac1 and Cdc42 was not sufficient to induce NF-κB or AP-1 transactivation. Together, our data provide strong evidence that Rac1 and Cdc42 play a crucial role in the activation of macrophages displayed by LAMPf.

DISCUSSION

Mycoplasmas have been demonstrated to be potent biological response modifiers by acting on immune cells, notably the monocyte lineage (6, 7). This property is likely to participate in the pathogenesis of Mycoplasma, especially in the autoimmune disorders associated with some species of this genus (4, 26). Mycoplasma-derived lipoproteins have been reported to play a crucial role in the activation of macrophages and the induction of proinflammatory cytokine secretion, but the signal transduction events responsible for this induction are not fully understood. Comprehension of the mechanisms by which mycoplasma-derived lipoproteins activate macrophages improves our understanding of the pathogenesis of these bacteria and helps to elucidate general mechanisms of macrophage activation leading to cytokine secretion. We have recently demonstrated...
that in both human and murine macrophages PTK (protein tyrosin kinase) and MAPK activation are required to transduce signals leading to cytokine production in response to M. fermentans-derived lipoproteins (8). Moreover, we have shown that LAMPf induce NF-κB, AP-1, and CREB (c-AMP response element-binding protein) nuclear translocation in these cells by measuring their respective DNA binding activities and stimulated NF-κB, AP-1, and c-fos transactivation (9). In the present paper we present evidence that small G proteins Rac1, Cdc42, and Rho play a crucial role in the signaling elicited by LAMPf and leading to macrophage activation. Cdc42 and Rac mediate the activation of JNK by LAMPf, whereas Rho seems to control the three MAPK cascades so far identified in mammals: ERK, JNK, and p38.

Few studies have addressed the involvement of the Rho family of small GTPases in the activation of MAPK pathways. In human kidney cells it has been reported that Cdc42 and
of Rac and Cdc42 abolished the activation by LAMPf. Although Rac and Cdc42 have been demonstrated to be sufficient to stimulate MAPKs in COS cells (10–12), the activated forms of Rac and Cdc42 failed to stimulate either MAPK or NF-κB and AP-1 transactivation in RAW 264.7 cells. This finding suggests that in macrophages Rac and Cdc42 may operate at some distance from the pathways being investigated, and the assistance of other signaling cascades could be necessary for a complete macrophage activation.

Although some cellular processes in macrophages that are controlled by small G proteins have been demonstrated to involve phosphoinositide 3-kinase (28, 29), the selective phosphoinositide 3-kinase inhibitors LY294002 and wortmannin did not modify the capacity of LAMPf to induce the secretion of proinflammatory cytokines (data not shown).

Despite the growing improvement of the picture of the *M. fermentans* signaling pathway, from this study and from our previous reports, the characterization of a putative receptors for mycoplasma lipoproteins remains elusive (7–9). However, we have provided strong evidence in the past that the activation of macrophages by mycoplasma lipoproteins is independent of CD14 (*i.e.* lipopolysaccharide receptor) (7, 8). Very recently we reported that a *M. fermentans*-derived synthetic lipopeptide (sMALP-2, for synthetic macrophage-activating lipopeptide-2) activates macrophages in a manner fully comparable with that of the membrane lipoprotein fraction (30). Therefore, sMALP-2 constitutes a valuable surrogate for identifying the macrophage membrane constituents responsible for the response elicited by LAMPf. This important issue is currently under investigation by our group.

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