A Rho Exchange Factor Mediates fMet-Leu-Phe-induced NF-κB Activation in Human Peripheral Blood Monocytes*

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We reported previously that fMLP stimulates NF-κB activation, and this function of fMLP requires small GTPase RhoA in human peripheral blood monocytes (Huang, S., Chen, L.-Y., Zuraw, B. L., Ye, R. D., and Pan, Z. K. (2001) J. Biol. Chem. 276, 40977–40981). Here we present evidence that RhoA associates specifically with the guanine nucleotide exchange factor Lbc in human peripheral blood monocytes stimulated with fMLP and that Lbc specifically catalyzes the guanine nucleotide exchange activity of RhoA in human peripheral blood monocytes. Cotransfection of the monocytic THP1 cells with lbc with a κB promoter reporter plasmid results in a marked increase in NF-κB-mediated reporter gene expression. Finally, Lbc-enhanced NF-κB activation is inhibited by a RhoA inhibitor, C3 transferase from Clostridium botulinum. A dominant-negative form of RhoA (T19N) also inhibited Lbc-enhanced reporter gene expression in a κB-dependent manner. These results indicate that guanine nucleotide exchange factor Lbc is a novel signal transducer for RhoA-mediated NF-κB activation in human peripheral blood monocytes stimulated with bacterial products.

Leukocyte infiltration is characteristic of inflammatory reaction to tissue injuries caused by trauma, invasion of foreign particles, ischemia-reperfusion, cancer, autoimmune diseases, and other conditions. This process begins with activation of leukocytes by a collection of cell-attracting chemicals termed chemoattractants. The bacterial tripeptide fMet-Leu-Phe (fMLP)\(^1\) is one of the most powerful chemoattractants, binds to seven transmembrane-spanning, G-protein-coupled receptors on monocytes and neutrophils and induces a variety of functional responses, including activation of the transcription factor NF-κB (1–3). However, the signaling mechanisms utilized by chemoattractants to activate NF-κB in human peripheral blood monocytes are poorly defined. fMLP stimulates peripheral blood mononuclear cells to express a defined set of gene products, including IL-1, IL-6, and IL-8. Pretreatment of the peripheral blood mononuclear cells with pertussis toxin abolishes fMLP-stimulated cytokine synthesis, suggesting that a G\(_4\) containing heterotrimeric G protein may mediate the process (4). Several recent studies have demonstrated activation of the transcription factor NF-κB by G protein-coupled receptors (5–9). NF-κB is of paramount importance to immune cell function because of its ability to activate the transcription of many proinflammatory immediate-early genes (10, 11). In leukocytes, NF-κB activation results in the transcription of immediate-early genes that encode IL-2, IL-6, IL-8, tumor necrosis factor-α, MCP-1, granulocyte/macrophage colony stimulating factor, as well as several adhesion molecules (11). We recently reported that chemoattractants (fMLP, PAF, C3a, and C5a) can activate the transcription factor NF-κB (3, 8, 12) and provided the evidence that fMLP-induced NF-κB activation is essential for proinflammatory cytokine synthesis in human peripheral blood monocytes (13). We further showed that fMLP stimulates small GTPase RhoA activity, and this activity in monocytes is required for NF-κB activation induced by fMLP (14).

The activation of Rho proteins is regulated by guanine nucleotide exchange factors (GEFs) that promote the transition between the inactive GDP-bound state and the active GTP-bound state of these GTPases. Rho-related exchange factors acquire tumorigenic activity by deletion of their N-terminal region, and most of them have been identified as potent oncodes isolated from different transformed cell lines. These putative GEFs share the structural arrangement of a 200-amino-acid motif, the Dbl homology domain, followed by a second putative signaling motif, the pleckstrin homology domain. Many GEFs have been identified for the Rho family of GTPases (Rho, Rac, and Cdc42), including: dbl, ost, lk, lbc, vav, and net (reviewed in Refs. 15 and 16). Among these, Lbc is reported to have GEF activity specific for RhoA in NIH 3T3 cells (17). Recently, an important role of Rho GTPases in gene expression has become apparent. Chang et al. (18) demonstrated that Rho activation is involved in AP-1-mediated transcription in Jurkat cells. Clostridium difficile toxin B, which inactivates Rho family proteins including RhoA, Rac, and Cdc42, has been reported to reduce the LPS-induced IL-8 expression in human umbilical vein endothelial cells (19). A recent report also indicated that Rho GTPases regulate c-fos transcription activation (20), that constitutively active Rho proteins can activate NF-κB (21), and that tumor necrosis factor-α-induced activation of NF-κB in NIH-3T3 cells is dependent on Cdc42 and RhoA (22). Despite an apparent role of the Rho GTPases in gene expression, relatively little is known about the molecular mechanisms of Rho activity and Rho signaling in gene transcription.

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‡ The abbreviations used are: fMLP, fMet-Leu-Phe; IL-1, interleukin 1; NF-κB, nuclear factor κB; GEF, guanine nucleotide exchange factor; GTPase, guanosine 5′-3′-O-thiotriphosphate or guanosine 5′-O-(3-thiotriphosphate); WT, wild type; LUC, luciferase; GST, glutathione S-transferase.
In this study, we therefore investigated the role of guanine nucleotide exchange factor in the signaling events that lead to activation of RhoA and NF-κB in fMLP-stimulated monocytes. We found that RhoA associates specifically with the guanine nucleotide exchange factor Lbc in monocytes stimulated with fMLP and that Lbc specifically catalyzes the guanine nucleotide exchange activity of RhoA. Cotransfection of the monocyte THP1 cells with lbc with a κB promoter reporter plasmid resulted in a marked increase in NF-κB-mediated reporter gene expression. Furthermore, lbc-induced NF-κB activation is blocked by a RhoA inhibitor or co-transfection with a RhoA dominant-negative mutant. These results suggest that fMLPs stimulate RhoA and NF-κB activation and that the guanine nucleotide exchange factor Lbc is involved in mediating this effect in human peripheral blood monocytes.

EXPERIMENTAL PROCEDURES

Reagents—DMLP was obtained from Sigma. Pertussis and cholera toxins were purchased from Calbiochem. The cDNA coding for Lbc was kindly provided by Y. Zheng (University of Tennessee). The polymerase chain reaction was used to insert the cDNA of the Lbc protein into the XbaI-BamHI cloning sites of the pGEX vector and pcDNA3 vector. The GST-RhoA was expressed in Escherichia coli. The RhoA dominant-negative (T19N) pcMV plasmids and recombinant Clostridium botulinum C3 transferase were obtained as described previously (21). Antibodies against Lbc, RhoA, Rac, and Cdc42 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Monocytes from Peripheral Blood—Heparinized human peripheral blood from healthy donors was fractionated on Percoll (Amersham Biosciences) density gradients. Mononuclear cells and neutrophils were initially separated by centrifugation through a 55%/40% discontinuous Percoll gradient. Monocytes were further prepared from the mononuclear cell population with gelatin/plasma coated flasks as described (8). The purity of monocytes was greater than 85–90% as determined by staining with an anti-CD14 monoclonal antibody (Coulter Immunology, Miami, FL), and cell viability was greater than 95% as measured by trypan blue exclusion. Monocytes were resuspended in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM; Irvine Scientific, Santa Ana, CA). GTP-γ-S Binding Assay—[35S]GTPγS binding of RhoA under the catalysis of Lbc was determined as described (23). RhoA was first loaded with GDP in loading buffer. The guanine nucleotide exchange reaction was initiated by mixing the GDP-loaded G protein with Lbc-containing immunoprecipitates (anti-Lbc antibody) or control immunoprecipitates (control antibody) in the reaction buffer with [35S]GTPγS and was terminated at various time points by dilution of the reaction mixture into ice-cold termination buffer. The amount of [35S]GTPγS bound to the G protein was finally quantified by filtration of the terminated reactions through nitrocellulose filter. Briefly, ~2 μg of GST-RhoA protein was incubated in 60 μl of loading buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.2 mM dithiothreitol, 100 μM AMP-PNP, and 10 μM GDP) for 5 min at room temperature. MgCl2 was added (5 mM) for 15 min. Aliquots (20 μl) of the GDP-loaded RhoA were mixed with 20 μl of Lbc-containing monocyte immunoprecipitates (anti-Lbc antibody) or control monocyte immunoprecipitates (control antibody) in the reaction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, MgCl2, AMP-PNP, 0.5 mg/ml bovine serum albumin, and 5 μM [35S]GTPγS) to initiate the exchange reaction at room temperature. The terminated reactions at various time points (0 min–20 min) were filtered immediately through the BA 85 nitrocellulose filters, and the radioactivity retained by the filter was measured by scintillation counting.

GDP Dissociation Assay—Approximately 2 μg of GST-RhoA protein was incubated in loading buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.2 mM dithiothreitol, 100 μM AMP-PNP, and 10 μM GDP) for 5 min at room temperature. MgCl2 was added (5 mM) for 15 min. Aliquots (20 μl) of the [3H]GDP-loaded RhoA were mixed with 20 μl of Lbc-containing monocyte immunoprecipitates (anti-Lbc antibody) or control monocyte immunoprecipitates (control antibody) in the reaction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 100 μM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 1 μM [3H]GTPγS) to initiate the exchange reaction at room temperature. The terminated reactions at various time points (0–20 min) were filtered immediately through the BA 85 nitrocellulose filters, and the radioactivity retained by the filter was measured by scintillation counting.

Immunoprecipitation and Immunoblotting—Monocyte lysates were incubated with an appropriate amount of antibody for 3 h and then precipitated following absorption onto protein A-Sepharose. Precipitates were washed three times, separated by SDS-PAGE, and transferred to Hybond-ECL nitrocellulose (Amersham Biosciences). Filter strips were incubated with primary antibody for 30 min at room temperature followed by addition of peroxidase-conjugated IgG at 1:10,000 for 30 min and analysis with enhanced chemiluminescence reagents (PerkinElmer Life Sciences).

Luciferase Activity Assay—The plasmid pCMV-κB-LUC (WT-IL-8-LUC) contains a κB site from the promoter region of the IL-8 gene, and a separate plasmid pCMV-κB-MU-LUC (MU-IL-8-LUC) has a nonfunctional κB site. Both constructs were kindly provided by Dr. N. Mackman (The Scripps Research Institute, La Jolla, CA) (24). The plasmid pCMVβ (Clontech) was used as a control for monitoring the transfection efficiency by the expression of β-galactosidase. THP1 cells were transiently transfected using diethylaminoethyl (DEAE)-dextran (25) and were cultivated for 48 h before a 4-h stimulation with medium or fMLP (100 ng/ml). Luciferase activity was determined by using the luciferase assay kit (Promega) and the Monolite 1000 luminometer (Analytical Luminescence, San Diego, CA).

RESULTS

Endogenous Lbc Interacts with RhoA in Monocytes Stimulated with fMLP—Guanine nucleotide exchange factor Lbc is reported to have GEF activity specific for Rho in NIH 3T3 cells. Lbc gene expression is strongly expressed in normal human peripheral blood leukocytes and HL-60 cells (26). We, therefore, hypothesized that guanine nucleotide exchange factor Lbc is a novel signal transducer for RhoA-mediated NF-κB activation in human peripheral blood monocytes stimulated with fMLP. To assess the role of Lbc in fMLP-induced RhoA-mediated NF-κB activation, we first examined the Lbc expression pattern in human peripheral blood leukocytes. We used an antibody that recognizes Lbc in order to detect the endogenous Lbc in human peripheral blood monocytes, neutrophils, and lymphocytes. Human blood lymphocytes contained little Lbc expression. In contrast, monocytes exhibited high endogenous Lbc expression, and neutrophils displayed low levels of Lbc (Fig. 1). We next examined whether the biological activity of the Lbc in monocytes is based on its ability to interact with low molecular weight GTP-binding proteins or whether Lbc can functionally bind to Rho family GTP-binding proteins in vivo. Monocytes (10 × 10^6) were stimulated with media (Fig. 2, lanes 1), or fMLP (100 nM) at 37°C for 10 min (Fig. 2, lanes 2), before the cells were lysed. Anti-Rho family GTPases (RhoA, Rac1, and Cdc42) antibodies (Santa Cruz Biotechnology) were used to immunoprecipitate Rho GTPases from the lysates, and the immunoprecipitates were detected by immunoblotting with anti-Lbc antibodies. Fig. 2 showed that the anti-RhoA (Fig. 2A), anti-Rac1 (Fig. 2B), and anti-Cdc42 (Fig. 2C) antibodies were used to immunoprecipitate GTPases from the lysates, and the precipitates were Western blotted with the anti-Lbc antibody. Only
RhoA was observed to co-precipitate with Lbc in fMLP-stimulated monocytes. As control experiments, we preincubated Rho GTPases antibodies with the blocking peptides (Santa Cruz Biotechnology), then immunoprecipitated as described above to test the specific interaction of fMLP-stimulated RhoA with Lbc (Fig. 2, lanes 3). These results indicate that Lbc is functionally associated with the Rho GTP-binding protein in monocytes stimulated with fMLP.

**Lbc Binding with RhoA in fMLP-stimulated Monocytes Involves fMLP Receptors Coupled to Pertussis Toxin-sensitive Heterotrimeric G Proteins—**We demonstrated previously that fMLP-induced RhoA activity in monocytes is mediated through fMLP receptors that are coupled to pertussis-sensitive heterotrimeric G proteins. We therefore examined whether the functional interaction between Lbc and RhoA is subject to inhibition by bacterial toxins that ADP-ribosylate the α-subunits of certain G-protein (Fig. 3). Monocytes were pretreated with pertussis toxin (Fig. 3, P.Tx) and cholera toxin (Fig. 3, C.Tx) separately and then stimulated with fMLP. Pertussis toxin (0.5 μg/ml) significantly reduced fMLP-induced interaction of Lbc and RhoA, whereas cholera toxin has no such inhibitory effect in monocytes. Thus, our results indicate that fMLP stimulates the interaction of Lbc and RhoA through fMLP receptors that are coupled to pertussis toxin-sensitive heterotrimeric G proteins.

**Lbc Functions as a Guanine Nucleotide Exchange Factor for RhoA in Monocytes Stimulated with fMLP—**The results presented above identify and confirm the interaction between Lbc and RhoA in monocytes under fMLP-stimulated conditions. We next used GTP binding assays to examine whether Lbc functions as a guanine nucleotide exchange factor for RhoA in monocytes stimulated with fMLP. Recombinant GST-RhoA were preloaded with [3H]GDP and incubated with immunoprecipitates (anti-Lbc antibody) from fMLP-stimulated monocytes for the indicated time before termination of the reactions by filtration through the nitrocellulose filter. The rate of dissociation of [3H]GDP from RhoA was stimulated ~6-fold by the Lbc-containing monocyte immunoprecipitates with anti-Lbc antibody (Fig. 4A). Similarly, there is an increase in the rate (~6-fold) of [35S]GTP·S binding to RhoA by Lbc-containing monocyte immunoprecipitates with anti-Lbc antibody (Fig. 4B). These results further confirmed and demonstrated that the interaction between RhoA and Lbc is functional importance and that Lbc specifically catalyzes the guanine nucleotide exchange activity of RhoA in human blood monocytes.

**RhoA Activity Is Required for Lbc-enhanced Reporter Gene Expression in a κB-dependent Manner—**We have previously demonstrated that fMLP stimulated RhoA activity, and this activity is required for fMLP-induced NF-κB activity in human monocytes (14). The results presented above showed that the interaction between RhoA and Lbc is functionally important and that Lbc specifically catalyzes the guanine nucleotide exchange activity of RhoA in human blood monocytes. These results suggest that Lbc may be involved in fMLP-induced NF-κB activation and cytokine gene expression in monocytes. To test this hypothesis, we examined the effect of Lbc on fMLP-induced NF-κB activation and cytokine gene expression in monocytes. Treatment of Lbc in THP1 cells induced luciferase activity in cells transfected with the WT-IL-8-LUC (Fig. 5A, lane 3) as compared with the vector control (lane 1, p < 0.01). In contrast, overexpression of Lbc in fMLP-stimulated THP1 cells (Fig. 5B, lane 2) did not cause a significant increase in luciferase activity in cells transfected with MU-IL-8-LUC (from which the κB site was deleted) as compared with fMLP-stimulated cells transfected with WT-IL-8-LUC (Fig. 5B, lane 4). The relationship among Lbc, RhoA, and subsequent NF-κB activation and cytokine gene expression was further explored using C3 transferase exoenzyme, an exotoxin produced by C. botulinum that specifically inhibits the RhoA small GTP-binding proteins but does not inhibit Rac or Cdc42 (24). Preincubation of THP1 cells with the recombinant C3 transferase exoenzyme (10 μg/ml, overnight) before co-transfection of Lbc with WT-IL-8-LUC reporter plasmid significantly inhibited the luciferase activity (Fig. 5C).

Further demonstration of the necessity for RhoA activity in Lbc-enhanced NF-κB activation was obtained by overexpressing a dominant-negative form of RhoA (RhoA-T19N). THP1 cells were co-transfected with an expression vector encoding a dominant-negative form of RhoA (RhoA-T19N) together with Lbc and WT-IL-8-LUC plasmid. Fig. 5D shows the effect of the dominant-negative RhoA mutant protein on Lbc-enhanced luciferase activity in THP1 cells. Co-transfection of the domi-
nent-negative RhoA plasmid with Lbc and the WT-IL-8-LUC plasmid significantly blocked Lbc-enhanced luciferase activity (Fig. 5D, lane 3). Taken together with the previous results, this strongly suggests that RhoA activity is required for Lbc-enhanced NF-κB activation and cytokine gene expression.

**DISCUSSION**

The Rho family of small GTP-binding proteins has been implicated in multiple signaling pathways leading to cytoskeleton reorganization (27), cell growth (28), and actin stress fiber and focal adhesion formation (29). Recently, studies both from other laboratories and our laboratory have shown that the Rho GTPases activate gene transcription factors (21, 22). The activation of Rho proteins is regulated by guanine nucleotide exchange factors, particularly Lbc, are an important part of the Rho GTPases (GEFs). Many GEFs have been identified for the Rho family of GTPases (RhoA, Rac, and Cdc42) (15, 16). Ost and Dbl have been shown to have GEF activity for both RhoA and Cdc42, whereas only Lbc demonstrates GEF activity that is specific for RhoA in NIH 3T3 cells (17). Lbc is a Dbl family protein and has several interesting features. First, it contains a Dbl homology domain, which may regulate the small G-proteins (30–32). Second, Lbc contains a pleckstrin homology domain, which may bind with phosphatidylinositol 3,4,5-trisphosphate, a product of phosphatidylinositol 3-kinase (33–36). Finally, Toksoz and Williams (26) showed that Lbc gene expression is strongly expressed in normal human peripheral blood leukocytes and HL-60 cells. We previously demonstrated that fMLP-induced NF-κB activation requires RhoA GTPase activity in human blood monocytes. We therefore investigated the role of guanine nucleotide exchange factor in the signaling events that lead to activation of RhoA and NF-κB in fMLP-stimulated human peripheral blood monocytes. We found that RhoA associates specifically with the guanine nucleotide exchange factor Lbc in monocytes stimulated with fMLP, suggesting that Lbc functions as a guanine nucleotide exchange factor for RhoA in human blood monocytes. These results were supported by the fact that cotransfection of the monocytic THP1 cells with lbc and a κB promoter plasmid results in a marked increase in NF-κB-mediated reporter gene expression. Furthermore, Lbc-enhanced NF-κB activation is blocked by inhibition of RhoA activity. These results suggest that fMLP stimulates RhoA and NF-κB activation and that the guanine nucleotide exchange factor Lbc may mediate this effect in human peripheral blood monocytes.

Although the activation of NF-κB has been extensively studied in cultured cell lines of hematopoietic lineage, the signal transduction pathways for this activation process are still incompletely understood. Results from recent studies have shown an important role of Rho GTPases in gene transcription and expression (18–21). Our previous report has also shown that fMLP stimulates NF-κB activation, and this function of fMLP requires small GTPase RhoA in monocytes (14). These earlier findings prompted us to investigate whether guanine nucleotide exchange factors, particularly Lbc, are an important part of the signaling molecule in RhoA-mediated NF-κB activation in human blood monocytes.

Our results indicated that the interaction between Rho and Lbc is functionally important and that Lbc specifically catalyzes...
the guanine nucleotide exchange activity of RhoA in human blood monocytes, suggesting that Lbc may play a role in RhoA-mediated downstream signaling. Our results also indicated that Lbc binding with RhoA in stimulated monocytes involves fMLP receptors coupled to pertussis-sensitive heterotrimeric G proteins, suggesting that these responses are transduced through the Gαi or Gαo class of heterotrimeric G proteins. These results were supported by the ability of Lbc to enhance NF-κB-mediated reporter gene expression. The role of Lbc in RhoA-mediated NF-κB activation was then confirmed by using C3 transferase, an inhibitor of RhoA, to abolish Lbc-enhanced NF-κB activation. The relationship among Lbc, RhoA, and subsequent NF-κB activation was further explored using a dominant-negative RhoA (RhoA-T19N) that inhibited Lbc-enhanced reporter gene expression. The observation that Lbc potently regulates NF-κB activation provides the evidence of a guanine nucleotide exchange factor that regulates gene transcription activation in human peripheral blood leukocytes. This function of Lbc was previously unidentified, although Lbc has been reported to have GEF activity specific for RhoA in NIH 3T3 cells (17). Taken together with the previous results, these data suggest that the guanine nucleotide exchange factor Lbc is a novel signal transducer for fMLP-induced RhoA-mediated NF-κB activation in human peripheral blood monocytes. The mechanisms of fMLP-dependent signaling increase the interaction of RhoA and Lbc, and the signaling molecules linking N-formyl peptide receptor-associated Gαi3 proteins and the Lbc, small GTPase RhoA, however, remain to be determined.

In summary, we have shown that RhoA associates specifically with the guanine nucleotide exchange factor Lbc in human peripheral blood monocytes stimulated with fMLP and that Lbc specifically catalyzes the guanine nucleotide exchange activity of RhoA in human peripheral blood monocytes. The interaction of Lbc and RhoA in fMLP-stimulated monocytes was inhibited by pertussis toxin but not cholera toxin, suggesting that the N-formyl peptide receptor-mediated responses are the results of coupling to the Gαi3 class of Gα proteins. Utilizing both a specific inhibitor and transient expression of a dominant-negative RhoA mutant, we further showed that RhoA activation is required for Lbc-enhanced reporter gene activity. These findings provide the first evidence of a guanine nucleotide exchange factor that regulates cytokine gene transcription in leukocytes. These findings also provide a physiological role for Lbc that contributes to inflammatory cytokine regulation in human peripheral blood monocytes stimulated with bacterial products.

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