Bacterial Lipopolysaccharide Induces Tyrosine Phosphorylation and Activation of Mitogen-activated Protein Kinases in Macrophages*

(Received for publication, January 22, 1992)

Steven L. Weinstein‡, Jasbinder S. Sanghera‡, Krista Lemke‡, Anthony L. DeFranco§, and Steven L. Pelech‡‡

From the Departments of Physiology and Microbiology and Immunology, University of California, San Francisco, California 94143-0553 and the Kinetic Biotechnology Corporation and the Biomedical Research Laboratory and the Department of Medicine, University of British Columbia, Vancouver, British Columbia V6T 2B5, Canada

Bacterial lipopolysaccharide (LPS) is a potent activator of antibacterial responses by macrophages. Following LPS stimulation, the tyrosine phosphorylation of several proteins is rapidly increased in macrophages, and this event appears to mediate some responses to LPS. We now report that two of these tyrosine phosphoproteins of 41 and 44 kDa are isoforms of mitogen-activated protein (MAP) kinase. Each of these proteins was reactive with anti-MAP kinase antibodies and comigrated with MAP kinase activity in fractions eluted from a MonoQ anion-exchange column. Following LPS stimulation, column fractions containing the tyrosine phosphorylated forms of p41 and p44 exhibited increased MAP kinase activity. Inhibition of LPS-induced tyrosine phosphorylation of these proteins was accompanied by inhibition of MAP kinase activity. Additionally, induction of p41/p44 tyrosine phosphorylation and MAP kinase activity by LPS appeared to be independent of activation of protein kinase C, even though phorbol esters also induced these responses. These results demonstrate that LPS induces the tyrosine phosphorylation and activation of at least two MAP kinase isozymes. Since MAP kinases appear to modulate cellular processes in response to extracellular signals, these kinases may be important targets for LPS action in macrophages.

The major outer membrane component of Gram-negative bacteria, lipopolysaccharide (LPS), is a potent activator of macropage responses involved in the host defense against infection (1, 2). LPS-activated macrophages secrete several immunoregulators that promote antibacterial responses by other cells such as interleukin-1, tumor necrosis factor, and macrophage inflammatory protein (MIP).

*This work was supported in part by a Grant AI20038 from the National Institutes of Health (to A. L. D.) and a grant from the National Cancer Institute of Canada (to S. L. P.). 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 1734 solely to indicate this fact.

**To whom correspondence and reprint requests should be addressed: G. W. Hooper Foundation, University of California, San Francisco, CA 94143-0552.

††Recipient of a Medical Research Council of Canada Scholarship Award.

1The abbreviations used are: LPS, lipopolysaccharide; MAP, mitogen-activated protein; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; p44MAP, 44-kDa MAP kinase encoded by sea star mpk gene; TBS, Tris-buffered saline; TBS-T, TBS containing 0.05% Tween 20; PMA, phorbol 12-myristate 13-acetate; MOPS, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate. arachidonic acid metabolites (3, 4). In addition, the combination of LPS and interferon-γ induce macrophages to differentiate to a highly bactericidal state. However, excessive LPS stimulation of macrophages and other cells occurring during severe bacterial infections can also lead to extensive tissue damage and septic shock (5). Thus, a better understanding of the mechanisms by which macrophages are activated by LPS could provide important insight into the regulation of the host response to bacterial infection.

We have investigated the early biochemical events that are triggered in macrophages by LPS. Previously, we reported that LPS increases protein tyrosine phosphorylation in murine macrophages and that this early signaling event appears to mediate some downstream macrophage responses to LPS (6). To extend these findings, we have attempted to identify the molecular components involved in the induced phosphorylation response. One such component is the macrophage cell surface protein, CD14. This protein has been shown to bind complexes consisting of LPS and serum LPS-binding protein and has been implicated in the cellular response to LPS (7). Inhibition of LPS binding to CD14 with anti-CD14 antibodies also inhibited LPS-induced tyrosine phosphorylation in human macrophages.2 This observation suggests that CD14 plays a role in mediating this signaling event. Also of interest are the identities of the proteins that undergo increased tyrosine phosphorylation following LPS stimulation. Among the most prominent tyrosine phosphorylated bands in LPS-stimulated macrophages are a series of 40–45-kDa proteins (6). These molecular masses are similar to those of a family of serine/threonine protein kinases known as mitogen-activated protein (MAP) kinases (8–10). MAP kinases appear to participate in the signal transduction pathways activated by a variety of extracellular ligands. These kinases have been shown in several cell types to be rapidly phosphorylated on tyrosine residues following cellular activation, and this modification contributes to the increased enzymatic activity of these proteins. The in vitro phosphorylation and activation of the 90-kDa ribosomal S6 protein kinase (11, 12) and the c-jun transcription factor (13) by MAP kinases suggest that they may regulate fundamental cellular processes.

Given the growing evidence that MAP kinases are important signal transduction components, we tested whether any of the tyrosine-phosphorylated bands observed in LPS-stimulated macrophages correspond to MAP kinases and whether these enzymes become activated following LPS treatment. In this report, we show that MAP kinase activity is increased following LPS treatment, and this response appears to occur as the result of tyrosine phosphorylation of at least two
different MAP kinase isozymes. Thus, MAP kinases are the first identified substrates for LPS-induced tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Preparations of LPS were purchased from List Biological Laboratories (Campbell, CA) and diphosphoryl lipid A was purchased from Ribi Immunochemical Research (Hamilton, MT). Synthetic lipid A (diphosphoryl, Escherichia coli type), (1→4)P2ATP, and the PY-20 antiphosphotyrosine monoclonal antibody were from ICN Biomedicals. The 4G10 antiphosphotyrosine monoclonal antibody was a gift from D. Morrison, B. Drucker, and T. Roberts (Dana-Farber Cancer Institute). Goat antirabbit IgG and goat antimouse IgG conjugated to alkaline phosphatase were procured from Bio-Rad. Sheep antimouse IgG was conjugated to horseradish peroxidase, and mouse antimouse IgG was obtained from Sigma. Complete and Incomplete Freund’s Adjuvant were purchased from Ribi Immunochemical Research (Hamilton, MT). Synbiolin (Department of Bio-Organic Chemistry, Genentech, San Francisco, CA). Complete and Incomplete Freund’s Adjuvant were from GIBCO. The bicinchoninic acid protein assay kit was purchased from Pierce Chemical (Rockford, IL). Other reagents were purchased from Sigma. Cell Culture, Stimulation, and Lysis—The murine macrophage cell line, RAW 264.7 (American Type Culture Collection, Rockville, MD), was cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum and 2 mM glutamine at 37°C in a 5% CO2/air mixture. For each experimental sample, 2 × 106 cells were seeded into a 150-mm dish in 20 ml of medium. The cells were then cultured for about 18 h to allow the cell number to approximately double. Cells were stimulated by the addition of the indicated activator for 15 min. In some experiments, prior to the addition of stimulators, cells were pretreated with 10 pg/ml herbimycin A for 4 h or 10 μM Compound 3 for 20 min. Following stimulation, cells were washed in situ with ice-cold phosphate-buffered saline containing 1 mM Na2VO4, then lysed in 2 ml of 20 mM MOPS, pH 7.2, 5 mM EGTA, 1% (w/v) Nonidet P-40, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 1 mM Na2VO4, and 1 mM phenylmethylsulfonyl fluoride for 20 min at 4°C. The detergent-insoluble material was pelleted by centrifugation (10,000 × g, 15 min, 4°C), and the soluble supernatant fraction was removed and stored at −80°C.

For immunoprecipitation of MAP kinase, cultures were seeded at 4 × 106 cells in a 100-mm dish in 10-ml medium. The cells were then cultured for about 18 h to allow the cell number to approximately double. Following stimulation, the cells were lysed in 0.5 ml of boiling 0.5% SDS, 1% (w/v) Nonidet P-40, 1% Triton X-100, 0.625% Nonidet P-40, 1.25 mM EDTA, 1.25 mM EGTA, 0.25% SDS, 1% dithiothreitol, 100 mM dithiothreitol, 10% glycerol, and 0.01% bromphenol blue. Samples were then separated on 12% SDS-polyacrylamide gels using the buffer system described by Laemmli (16). Following electrophoresis, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 30 min and then the proteins were transferred to nitrocellulose for 4 h at 0.5 A. Subsequently, the nitrocellulose membrane was blocked with Tris-buffered saline (TBS) containing 3% gelatin or 2% bovine serum albumin for 2 h at room temperature. The membrane was washed twice with TBS containing 0.05% Tween 20 (TBST) for 5 min before overnight incubation with rabbit polyclonal anti-MAP kinase antibodies or the mouse monoclonal PY-20 antiphosphotyrosine antibody (in 1% gelatin-TBST; 1:1000 dilution) or the mouse monoclonal 4G10 antiphosphotyrosine antibody (1:3 diluted culture supernatant in TBST). The next day, the membrane was washed twice with TBST before incubation with the secondary antibody (sheep antimouse IgG conjugated to horseradish peroxidase or goat antimouse IgG conjugated to alkaline phosphatase in 1% gelatin-TBST; 1:3000 dilution) for 2 h at room temperature. The membrane was rinsed with twice washes of TBST, followed by one wash with TBS prior to color development with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml nitro blue tetrazolium in 0.1 M NaHCO3, 10 mM MgCl2, pH 9.8. The color development was continued for 5 min to 4 h to give the desired darkness, and the reaction was stopped by rinsing the membrane in a large volume of water.

Immunoprecipitation of MAP Kinase—Cell lysates in 0.5% SDS, 10 mM Tris, pH 7.3, 1.25 mM dithiothreitol were diluted 1:5 with 12.5 mM Tris, pH 7.3, 187.5 mM NaCl, 1.25% deoxycholate, 1.25% Triton X-100, 0.625% Nonidet P-40, 1.25 mM EDTA, 1.25 mM EGTA, 0.25 mM Na2VO4, 0.25 mM phenylmethylsulfonyl fluoride. Diluted lysates (1.5 ml) were precleared with 5 μg of affinity-purified rabbit anti-mouse IgG and 40 μl of packed protein A-Sepharose beads for 30 min. The lysates were then incubated with a combination of 5 μg of a mouse monoclonal anti-MAP kinase antibody and 5 μl of erk1-CT anti-MAP kinase polyclonal antibodies for 2 h followed by the addition of 3 μg of affinity-purified rabbit antiamouse IgG for 1 h. Immune complexes were precipitated by transferring the lysates to tubes coated with a-Sephadex A-40 (Pharmacia Fine Chemicals, Piscataway, NJ). Precipitates were washed three times with 500 μl of incubation buffer (0.5 mM EDTA, 10 mM Tris, 50 mM NaCl) and once with 10 mM Tris, 50 mM NaCl for 1 h. All incubations were performed at 4°C. The beads were washed once with 1 ml of wash buffer (10 mM Tris, pH 7.3, 2 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.25 mM Na2VO4, 0.2 mM phenylmethylsulfonyl fluoride) and twice with wash buffer containing 150 mM NaCl. The beads were resuspended in 40 μl of 2% concentrated SDS sample buffer and boiled 10 min. The supernatant fraction was resolved on a SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The tyrosine phosphoproteins were visualized by immunoblotting with the 4G10 antiphosphotyrosine monoclonal antibody followed by sheep antiamouse IgG-horseradish peroxidase antibodies (1:15,000 dilution in TBST, 1 h, 25°C) and an enhanced chemiluminescence detection system used as directed by the manufacturer. The membrane was stripped (2% SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, 50% methanol, 5% acetic acid, 0.1% sodium dodecyl sulfate, and 0.05% Tween 20) and reprobed with a monoclonal anti-MAP kinase antibody (15,000 dilution in TBST, overnight, 4°C), followed by sheep antiamouse IgG-horseradish peroxidase antibodies (1:15,000 dilution in TBST, 1 h, 25°C). The immunoreactive proteins were then visualized on film by chemiluminescence.

RESULTS

LPS- and PMA-induced Tyrosine Phosphorylated p41 and p44 Correspond to MAP Kinase Isozymes—LPS rapidly increases the tyrosine phosphorylation of several proteins in the macrophage cell line, RAW 264.7, including four bands of 41, 42, 43.5, and 44 kDa as measured by antiphosphotyrosine immunoblotting (6) and Fig. 1, A and F). In addition, treat-
ment of these cells with phorbol 12-myristate 13-acetate (PMA) induces tyrosine phosphorylation of the 41- and 44-kDa proteins. To test whether any of these LPS- and PMA-induced bands correspond to MAP kinase isozymes, parallel immunoblots were probed with a panel of antibodies specific for MAP kinases (Fig. 1, B-E). These antibodies were raised against the purified sea star MAP kinase, p44K, or against peptides derived from p44K or the rat ERK 1 gene product. These antibodies have been shown to detect a 41–42-kDa isoform of MAP kinase as well as a 43–44-kDa isoform in 3T3 fibroblasts (11) and in other cell types. In RAW 264.7 cells, these antibodies detected two isoforms of MAP kinase which comigrated with the 41- and the 44.5-kDa tyrosine phosphoproteins induced by LPS and PMA. In contrast, the anti-MAP kinase antibodies did not react with bands corresponding to the 42- or the 45-kDa tyrosine phosphoproteins induced by LPS but not PMA. Thus, RAW 264.7 cells have at least two isoforms of MAP kinase and each isoform comigrates on SDS-polyacrylamide gels with a protein that becomes tyrosine-phosphorylated following treatment with LPS or PMA. Interestingly, the 41-kDa immunoreactive MAP kinase from LPS- and PMA-stimulated cells migrated slightly more slowly on the SDS-polyacrylamide gel than the corresponding protein from unstimulated cells. A similar mobility shift has been reported for a 42-kDa MAP kinase isozyme in Xenopus laevis oocytes following progesterone treatment (17) and in platelet-derived growth factor-stimulated fibroblasts (18). It has been suggested that this mobility shift may be a consequence of increased phosphorylation.

To further assess the relationship between the 41- and 44-kDa proteins detected by antiphosphotyrosine antibodies and by anti-MAP kinase antibodies, RAW 264.7 cell lysates were immunoprecipitated with a mixture of monoclonal and polyclonal anti-MAP kinase antibodies, and the resulting precipitated and unprecipitated fractions were separated on a SDS-polyacrylamide gel and immunoblotted with antiphosphotyrosine antibodies. Following anti-MAP kinase immunoprecipitation, the 44-kDa tyrosine phosphoprotein was completely depleted and the 41-kDa phosphoprotein was partially depleted from the cell lysate (Fig. 2A). This result indicates that both of these proteins were recognized by the anti-MAP kinase antibodies. Depletion of these two proteins was specific as other tyrosine phosphoproteins were not affected. Moreover, when these blots were stripped and reprobed with anti-MAP kinase antibodies, the 41- and 44-kDa MAP kinase isozymes were found to be depleted to the same extent as the corresponding tyrosine phosphoproteins (Fig. 2B). These results suggest that the 41- and 44-kDa tyrosine phosphoproteins induced by LPS are isoforms of MAP kinase.

The anti-MAP kinase immunoprecipitated fractions were also analyzed by immunoblotting with antiphosphotyrosine antibodies (Fig. 2A). We found that the 41- and 44-kDa MAP kinase isoforms were phosphorylated on tyrosine to a greater extent after stimulation with LPS. Since similar amounts of these MAP kinases were immunoprecipitated from the unstimulated and the LPS-stimulated cell lysates (Fig. 2B), these results directly demonstrate that LPS treatment increases the tyrosine phosphorylation of the 41- and 44-kDa MAP kinases.

**LPS Activates MAP Kinase Isoforms**—Since LPS increases the tyrosine phosphorylation of two MAP kinase isoforms, and as tyrosine phosphorylation is thought to be a critical event activating these enzymes, we tested whether LPS alters MAP kinase activity. RAW 264.7 cell lysates were fractionated by MonoQ anion-exchange chromatography, and the resulting column fractions were analyzed for MAP kinase activity and immunoblotted with antiphosphotyrosine antibodies or anti-MAP kinase antibodies. LPS treatment resulted in a large increase in MAP kinase activity as assessed by the phosphorylation of MBP, a standard substrate of MAP kinase (Fig. 3A). The increased activity was partially resolved.

---

3 S. L. Pelech, unpublished experiments.
of these phenomena are thought to be indicative of increased phosphorylation of these proteins (17–20).

Comparison of the immunoblotting data and the MAP kinase activity profile revealed that column fractions with elevated activity contained both the tyrosine-phosphorylated, 41- and 44-kDa isoforms of MAP kinase. Thus, the contribution of each isozyme to a particular activity peak could not be determined. In any case, LPS increased MAP kinase activity in RAW 264.7 cells, and this effect correlated with the induced tyrosine phosphorylation of at least two MAP kinase isoforms.

Similar increases in MAP kinase activity were observed following stimulation of RAW 264.7 cells with smooth or rough forms of LPS as well as purified bacterial or synthetic lipid A preparations (Fig. 4). Thus, different biologically active forms of LPS activate MAP kinases and this response appears to be lipid A-dependent, as is the case for almost all the effects of LPS on macrophages (4).

Herbimycin A Inhibits LPS-induced Activation of MAP Kinases—The enzymatic activity of MAP kinase isoforms is regulated in part by phosphorylation on tyrosine residues (21). Since LPS induces the tyrosine phosphorylation of the 41- and 44-kDa MAP kinase isoforms, LPS may modulate MAP kinase activity by inducing p41/p44 MAP kinase tyrosine phosphorylation. To test this possibility, we examined the effect of preventing LPS-induced tyrosine phosphorylation of p41/p44 on the MAP kinase activity. Previously, we reported that herbimycin A, a protein tyrosine kinase inhibitor (22–24), completely blocks tyrosine phosphorylation of all the proteins modulated by LPS including the 41- and 44-kDa proteins (6). Herbimycin A pretreatment also inhibited LPS-induced MAP kinase activity (Fig. 5A). In addition, the LPS-induced mobility shift of the 41-kDa immunoreactive MAP kinase isoform and the delayed elution of the 44-kDa isoform were absent in cells pretreated with herbimycin A (Fig. 5, C and D). Together, these results demonstrate a strong correlation between the tyrosine phosphorylation status of the 41- and 44-kDa MAP kinase isoforms and the level of MAP kinase activity in RAW 264.7 macrophages.

PMA Stimulates MAP Kinase Activity—Similar to the effects of LPS on RAW 264.7 macrophages, PMA treatment...
also induces the tyrosine phosphorylation of 41- and 44-kDa species that correspond to MAP kinase isoforms (Fig. 1).

Therefore, we tested whether MAP kinase activity is elevated in PMA-stimulated RAW 264.7 cells. PMA stimulation strongly increased the amount of MAP kinase activity with two peaks of activity partially resolved by MonoQ chromatography (Fig. 6A). A large peak of activity eluted in fractions 28–33 and a second, smaller peak that resolved as a shoulder of the first peak eluted in fractions 34–40. Thus, like LPS, PMA appeared to activate at least two isoforms of MAP kinase. Interestingly, following PMA treatment two 44-kDa MAP kinase isoforms with different MonoQ elution patterns were detected on antiphosphotyrosine and anti-MAP kinase immunoblots (Fig. 6, B–D). One of these proteins eluted from the column in the same fractions as p44 MAP kinase from unstimulated cells and appeared in lane 4 on the blots. The other protein was more strongly bound to the column and was detected in lanes 7 and 8. A similar phenomenon has been observed in nerve growth factor-stimulated PC12 cells (20) and in insulin-treated rat fibroblasts (19). It is unclear whether this observation results from the detection of two different MAP kinase isoforms of similar molecular weight or two different forms of the same isoform. As mentioned earlier, LPS also induced some increased retention of p44 to the MonoQ column. However, LPS stimulation did not result in the appearance of two distinct 44-kDa MAP kinase species. Thus, the effects induced by LPS and PMA on the 44-kDa isoform(s) of MAP kinase were not identical in RAW 264.7 cells.

**Protein Kinase Inhibitors Differentially Affect LPS- and PMA-stimulated MAP Kinase Activation**—To further investigate the mechanism by which LPS and PMA activate MAP kinase isoforms, we examined the effect of herbimycin A on the response triggered by PMA. Herbimycin A treatment, which completely blocked LPS-induced tyrosine phosphorylation and activation of MAP kinase, only weakly inhibited PMA induction of these responses (Fig. 7, A, C, E, G). Since, the targets of herbimycin A action are thought to be protein tyrosine kinases, the activation of MAP kinase by LPS appears to involve a herbimycin-sensitive tyrosine kinase, whereas the PMA-induced response does not.

The results with a protein kinase C inhibitor provide further evidence that LPS-induced activation and PMA-induced activation of MAP kinase are mechanistically different. We found that pretreatment with the staurosporine analog, Compound 3 (25), inhibited PMA-induced tyrosine phosphorylation of p41 and p44 and also inhibited activation of MAP kinase (Fig. 7, A, B, D, and F). This result was expected since phorbol esters are thought to exert their effects on cells by activating protein kinase C. In contrast, these LPS-induced responses were insensitive to Compound 3. (Fig. 8). Thus, the mechanism by which LPS activates MAP kinases in RAW 264.7 cells does not appear to be dependent on protein kinase C.

**DISCUSSION**

LPS stimulation of macrophages results in the increased tyrosine phosphorylation of several proteins. Here, we report that two of the induced tyrosine phosphoproteins, of 41 and 44 kDa, correspond to isoforms of MAP kinase. Each of these proteins was immunoreactive with anti-MAP kinase antibodies and could be partially depleted from cell lysates by immunoprecipitation with these antibodies. In addition, both pp41 and pp44 coeluted with MAP kinase activity following...
Fig. 7. Effect of herbimycin A and Compound 3 on PMA activation of MAP kinases. MonoQ chromatography of RAW 264.7 cell protein from cells exposed to 100 nM PMA for 15 min in the absence (●) and presence of herbimycin A (△) or Compound 3 (○) was performed, and the MAP kinase activity of column fractions using MBP as a substrate is shown in A. The combined column fractions from PMA + herbimycin A-treated cells (C, E, G) and PMA + Compound 3-treated cells (B, D, F), designated by outlined numbers in A, were immunoblotted with the PY-20 antiphosphotyrosine antibody (B, C) or anti-MAP kinase antibodies, anti-p44 MAP antibodies (D, E), or anti-erk1-CT peptide antibodies (F, G). The unstimulated and PMA-stimulated controls are shown in Figs. 3 and 6, respectively. The position of the shifted form of the 41-kDa MAP kinase is indicated with an arrow. Similar results were obtained in duplicate experiments.

MonoQ chromatography. Together, these results show that the 41- and 44-kDa proteins, whose tyrosine phosphorylation is induced by LPS, are isozymes of MAP kinase.

To date, at least four highly related MAP kinases have been described in a variety of species by biochemical, immunological, and molecular cloning data (8–10). One isoform has a mass of 42 kDa and is tyrosine-phosphorylated in response to mitogenic stimulation in a wide variety of cells. This MAP kinase isoform has been designated p42\textsuperscript{mapk} and corresponds to the ERK 2 gene product (20, 26). A slightly larger MAP kinase isoform (43–44 kDa) exhibits induced tyrosine phosphorylation often in parallel with increased phosphorylation of the 42-kDa isoform and is thought to correspond to ERK 1 (10). The existence of additional MAP kinase isoforms has been inferred from the molecular cloning of a third sequence-related cDNA (ERK 3; predicted molecular mass, 63 kDa) (20) and from the immunoblotting of a 45-kDa polypeptide (ERK 4) with anti-ERK 1 antibodies (20). The 41-kDa MAP kinase isoform from RAW 264.7 macrophages behaves most similarly to p42\textsuperscript{mapk}/ERK 2. In addition to the similar molecular mass, both MAP kinases exhibit slightly decreased mobility on SDS-polyacrylamide gels, following cellular activation. Moreover, anti-p44\textsuperscript{map} antibodies recognized the 41-kDa isoform more efficiently after LPS or PMA stimulation of RAW 264.7 cells. This property was previously observed with p42 MAP kinase from Xenopus oocytes (17). The 44-kDa MAP kinase isoform from RAW 264.7 cells behaves like p44 MAP kinase from PC 12 cells (20) and rat fibroblasts (19). For each of these 44-kDa isoforms, cellular stimulation leads to stronger binding of the 44-kDa protein to a MonoQ column. Although positive identification will require additional experiments, it seems likely that the two isoforms of MAP kinase activated by LPS in macrophages correspond to the two major MAP kinase isoforms seen in other cell types.

LPS-induced tyrosine phosphorylation of the two MAP kinase isoforms in RAW 264.7 macrophages was accompanied by increased MAP kinase activity. This activity could be partially resolved into two peaks by MonoQ chromatography suggesting that at least two MAP kinase isoforms were activated by LPS. Each of the column fractions with elevated MAP kinase activity contained tyrosine phosphorylated forms of the 41- and 44-kDa MAP kinase isozymes. Since tyrosine phosphorylation of MAP kinases is necessary for the activation of these proteins, both the 41- and 44-kDa isoforms are likely to contribute to the observed MAP kinase activity. The loss of MAP kinase activity which accompanied inhibition of induced tyrosine phosphorylation of these proteins is consistent with this interpretation. Moreover, the antiphosphotyrosine blots of the active column fractions did not reveal the presence of any other tyrosine phosphoproteins. This observation makes it unlikely that additional MAP kinase isozymes which were not detected by our MAP kinase antibodies were
responsible for the observed MAP kinase activity. Therefore, LPS appears to modulate MAP kinase activity in RAW 264.7 cells by inducing the tyrosine phosphorylation of the 41- and 44-kDa MAP kinase isoforms. Since phosphorylation on threonine residues has been shown to be necessary for the full activation of MAP kinase (22, 27), LPS may additionally modulate MAP kinase activity by increasing threonine phosphorylation of these proteins. Alternatively, MAP kinases in RAW 264.7 cells may be phosphorylated on regulatory threonine residues prior to LPS stimulation.

The mechanism by which LPS treatment increases the tyrosine phosphorylation of MAP kinases is not clear. Recent evidence from several groups has indicated that MAP kinases can autophosphorylate on tyrosine residues as well as threonine residues (14, 28–30). Thus, LPS-induced tyrosine phosphorylation of MAP kinase isoforms may occur by a LPS-stimulated autophosphorylation mechanism, independent of other protein tyrosine kinases. Alternatively, increased MAP kinase tyrosine phosphorylation could be a consequence of LPS-activated protein tyrosine kinases.

The ability of herbimycin A to inhibit both LPS-induced tyrosine phosphorylation and activation of the MAP kinase proteins suggests that an activated protein tyrosine kinase is necessary for these LPS responses. While the mechanism of action of herbimycin A is not completely understood, this inhibitor appears to inactivate protein tyrosine kinases by irreversibly binding to thiol groups in the affected kinases (22, 23). In addition, herbimycin A binding to these kinases promotes their degradation (22, 31). Since the amount of MAP kinases detected by immunoblotting did not change following herbimycin A treatment, these kinases may not be targets of this inhibitor. Moreover, PMA-induced MAP kinase tyrosine phosphorylation and activation was only weakly affected by herbimycin A treatment. This result demonstrates that herbimycin A does not directly inhibit MAP kinase. Thus, it seems most likely that herbimycin A inhibits an upstream protein tyrosine kinase that is necessary for the LPS-induced tyrosine phosphorylation of MAP kinases.

The effects of herbimycin A on LPS- and PMA-induced MAP kinase activity also suggest that MAP kinases may mediate some of the antibacterial responses of macrophages. First, herbimycin A, which prevented the LPS-stimulated increase in MAP kinase activity, also inhibits the release of arachidonic acid metabolites from LPS-treated RAW 264.7 macrophages (6). Arachidonic acid metabolites are potent inflammatory mediators, and their secretion by macrophages is characteristic of the activated state. In contrast to the results with LPS, herbimycin A did not strongly inhibit PMA-stimulated MAP kinase activation or the release of arachidonic acid metabolites (Fig. 7 and Ref. 6). Thus, the induction of MAP kinase activity by LPS and PMA appears to be correlated with the appearance of at least some downstream macrophage responses. A further indication of the relationship between MAP kinase activity and macrophage activation was provided by the results obtained with the protein kinase C inhibitor, Compound 3, in PMA-stimulated cells. Compound 3, which blocked PMA-induced MAP kinase activation, also inhibited the release of arachidonic acid metabolites (data not shown). However, the results with RAW 264.7 macrophages pretreated with Compound 3 and then stimulated with LPS do not fit this pattern. Compound 3 treatment, which did not inhibit LPS induction of MAP kinase activity, did inhibit LPS stimulation of arachidonic acid metabolite release (data not shown). Thus, in this case, an activated macrophage response was not triggered despite the induction of MAP kinase activity by LPS. This result, however, is still consistent with the hypothesis that MAP kinases participate in LPS-stimulated signal transduction. For example, one obvious explanation is that protein kinase C, or another protein kinase that is inhibited by Compound 3, participates in the LPS signaling pathway downstream of MAP kinase activation. Alternatively, the release of arachidonic acid metabolites induced by LPS in RAW 264.7 macrophages may require the generation of two independent intracellular signals, one provided by MAP kinases and the other by protein kinase C. Clearly the experiments presented here do not prove that MAP kinases mediate macrophage responses to LPS, but the results with the protein kinase inhibitors are consistent with this hypothesis.

The precise function of MAP kinases in macrophages and in other cells is, however, not yet known. Several proteins have been found to be efficient in vitro substrates of MAP kinases, and these may be indicative of MAP kinase function in vivo. For example, phosphorylation of microtubule-associated protein 2 by MAP kinases (32, 33) may alter the cytoskeleton and could provide a molecular mechanism for the morphological changes induced by LPS in macrophages. Similarly, MAP kinases can phosphorylate and activate both the S6 ribosomal protein kinase (11, 12) and the c-jun product (13), proteins that are involved in the regulation of translation and transcription, respectively. If MAP kinases phosphorylate these targets in vivo, these kinases may contribute to the altered expression of LPS-modulated proteins. Since many of the responses triggered by LPS in macrophages depend on transcription and translation, LPS activation of MAP kinases could be a critical part of the mechanism by which LPS induces responses in macrophages. Despite the incomplete understanding of the role of MAP kinases in cells, our results suggest that these kinases could be very important targets of LPS action in macrophages and lend support to the hypothesis that induced protein tyrosine phosphorylation is part of the signal transduction pathway that mediates macrophage responses to LPS.

Acknowledgments—Ian Curk-Lewis, Philip Owen, Greg Radigan, and Peter Borowski aided in the synthesis and purification of the peptides. Faye Chow and Michael Williams provided valuable technical assistance in the preparation of the antipeptide antibodies. We thank Vivien Chan, Ruth Giobus, Debbie Law, and Tracy Stevens for critical reading of the manuscript.

REFERENCES
1. Morrison, D. C., and Ryan, J. L. (1979) Adv. Immunol. 28, 293–450
2. Pabst, M. J., and Johnston, R. S. (1989) in Handbook of Immunobiology (Bienen, P. M., and Murphy, R. C., eds) Vol. 6, pp. 391–395, Elsevier, New York
3. Adams, D. O., and Hamilton, T. A. (1984) Annu. Rev. Immunol. 2, 263–318
4. Morrison, D. C., and Ryan, J. L. (1987) Annu. Rev. Med. 38, 417–432
5. Barriere, S. L., Ogibene, F. P., Summer, W. R., and Young, L. S. (1991) Patient Care 25, 86–109
6. Weinstein, S. L., Gold, M. R., and DeFranco, A. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4146–4150
7. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) Science 249, 1431–1433
8. Pelch, S. L., Sanghera, J. S., and Daga-Makin, M. (1990) Biochim. Biophys. Acta 1030, 389–398
9. Sturgill, T. W., and Wu, J. (1991) Biochim. Biophys. Acta 1092, 350–357
10. Cobb, M. H., Boulton, T. G., and Robbins, D. J. (1991) Cell Regul. 2, 965–978
11. Chung, J., Pelech, S. L., and Blenis, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4961–4965
12. Sturgill, T. W., Rey, I. B., Ekesson, E., and Maller, J. L. (1988) Nature 338, 713–718
13. Pulverer, B. J., Kyriakis, J. M., Arvuch, J., Nikolakaki, E., and Woodgett, J. R. (1991) Nature 349, 670–674
14. Sanghera, J. S., Paddon, H. B., Bader, S. A., and Pelech, S. L. (1996) J. Biol. Chem. 265, 52–57
15. Sanghera, J. S., Paddon, H. B., and Pelech, S. L. (1991) J. Biol. Chem. 266, 6700–6707
16. Laemmli, U. K. (1970) Nature 227, 680–685
17. Posada, J., Sanghera, J., Pelech, S., Averfeld, R., and Cooper, J. A. (1991) Mol. Cell Biol. 11, 2517–2528
18. Cooper, J. A., and Hunter, T. (1985) Mol. Cell Biol. 5, 3304–3309
LPS Activation of MAP Kinases in Macrophages

19. Boulton, T. G., and Cobb, M. H. (1991) *Cell Regul.* 2, 357-371
20. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) *Cell* 65, 663-675
21. Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990) *Nature* 343, 651-655
22. Fukazawa, H., Li, P.-M., Yamanoto, C., Murakami, Y., Mizuno, S., and Uehara, Y. (1991) *Biochem. Pharmacol.* 42, 1661-1671
23. Uehara, Y., Fukazawa, H., Murakami, Y., and Mizuno, S. (1989) *Biochem. Biophys. Res. Commun.* 163, 803-809
24. Uehara, Y., and Fukazawa, H. (1991) *Methods Enzymol.* 201, 370-379
25. Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D., and Wilkinson, S. E. (1989) *FEBS Lett.* 259, 61-63
26. Her, J. H., Wu, J., Rall, T. B., Sturgill, T. W., and Weber, M. J. (1991) *Nucleic Acids Res.* 19, 3743
27. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shah�nowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) *EMBO J.* 10, 885-892
28. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D., Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlien, R. L., Cobb, M. H., and Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 6142-6146
29. Wu, J., Rossomando, A. J., Her, J. H., Del Vecchio, R., Weber, M. J., and Sturgill, T. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 9308-9312
30. Crews, C. M., Alessandri, A. A., and Erikson, R. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 8845-8849
31. Jones, C. H., Fletcher, M. C., Leibetter, J. A., Shieven, G. L., Siegel, J. N., Phillips, A. P., and Samelson, L. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 7722-7726
32. Gotoh, Y., Nishida, E., Matsuda, S., Shina, N., Kosako, H., Shikawa, K., Akiyama, T., Ohta, K., and Sakai, H. (1991) *Nature* 349, 251-254
33. Ray, L. B., and Sturgill, T. W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1502-1506