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Bacterial lipopolysaccharides, phorbol myristate acetate, and zymosan induce the myristoylation of specific macrophage proteins

(protein acylation/arachidonic acid metabolism/protein kinase C/signal transduction/membrane attachment)

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ABSTRACT **We demonstrate stimulus-dependent incorporation of exogenously added [^{3}H]myristic acid into specific macrophage proteins. In control unstimulated cells an 18-kDa protein is the major acylated species. In cells incubated with bacterial lipopolysaccharide (LPS), or its monoacyl glucosamine phosphate derivative, fatty acid is incorporated into proteins with molecular mass of 68 kDa and a doublet of approximately 42–45 kDa. Phorbol 12-myristate 13-acetate (PMA) or a phagocytic stimulus (zymosan) promotes the acylation of a similar array of proteins. However, PMA and zymosan also promote the myristoylation of unique proteins of 92 and 50 kDa. The fatty acid associated with each of the acylated proteins is myristic acid. The myristate is probably linked to the proteins through amide bonds, since it is not released by treatment with hydroxylamine. Palmitate and arachidonate are not incorporated into proteins in the same manner. Temporal analysis revealed that LPS-induced proteins are myristoylated by 30 min, while the 50-kDa protein myristoylated in response to PMA is labeled later. Most myristoylated proteins appear to be associated with the membrane fraction. Macrophages from C3H/HeJ mice, which do not respond to LPS, do not show any LPS-dependent protein acylation. Interestingly, zymosan and PMA induce the myristoylation of the 50-kDa protein in C3H/HeJ macrophages, but not the acylation of the 68-kDa and 42-kDa doublet species. We suggest that myristoylation of specific proteins is an intermediary in the capacity of LPS, PMA, and zymosan to alter macrophage functions such as arachidonic acid metabolism.**

A major mechanism whereby macrophages mediate inflammation is through the secretion of arachidonic acid (20:4) metabolites (1). When murine resident peritoneal macrophages interact with zymosan particles or with phorbol 12-myristate 13-acetate (PMA) they secrete 20:4 metabolites (2, 3). We have recently shown that treatment of cells with bacterial lipopolysaccharide (LPS) increases the maximal amount of 20:4 release induced by zymosan or PMA and eliminates the lag phase of the response seen with zymosan or PMA alone (4). The active moiety of LPS, lipid A, contains a 3-OH-myristic acid moiety that has been shown to be important in LPS-induced responses (5, 6). Since acylation of select proteins has been described in several cell types (7, 8), we considered the possibility that the acylation of macrophage proteins with the 3-OH-myristic acid moiety of LPS is involved in the effect of LPS on 20:4 release by macrophages. Our first approach was to determine whether stimulation of macrophages resulted in the incorporation of exogenous [^{3}H]myristic acid into specific proteins.

Two general protein acylation reactions have been reported. The first involves the palmitoylation of proteins via ester bonds (review, ref. 9) and the other the amide linkage of myristic acid to proteins (10–14). Many of the proteins that have been shown to have the capacity to be myristoylated are important in cellular regulation, including the catalytic subunit of the cAMP-dependent protein kinase (10), calcineurin B (a component of a calmodulin-binding phosphatase) (11), the pp^{56} tyrosine kinase (12), and the pp60^{src} tyrosine kinase (13, 14). Although the function of the myristic acid moiety in these acylated proteins is unknown, it has been shown in the case of the pp60^{src} to promote the association of the tyrosine kinase with membranes and is required for its transformation properties (13, 14).

In this report we demonstrate that specific proteins are acylated with exogenous myristic acid when macrophages are stimulated with LPS, zymosan, or PMA.

MATERIALS AND METHODS

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of specific pathogen-free female ICR mice (Trudeau Institute, Saranac Lake, NY) or from C3H/HeJ mice (The Jackson Laboratory) as previously described (15). Peritoneal cells (approximately 9×10^6 per ml) in α modified minimal essential medium (α-MEM; GIBCO) containing 10% fetal calf serum were cultured in 35-mm-diameter plastic culture dishes (1 ml per dish). After 2 hr at 37°C in 95% air/5% CO_2, cultures were washed three times in calcium- and magnesium-free phosphate-buffered saline (PD) to remove nonadherent cells. The cells were then incubated overnight in α-MEM containing 10% fetal calf serum.

Myristoylation of Macrophage Proteins. Macrophages cultured at a density of approximately 3×10^6 cells per 35-mm culture dish were washed four times with PD and incubated for the indicated times in 1 ml of α-MEM containing [9,10-^{3}H(N)]myristic acid (20–40 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) and the specified stimuli. Stimuli included PMA (Sigma), *Escherichia coli* K-12 LPS (List Biologicals, Campbell, CA), monoacyl glucosamine phosphate (MAGP) (Lipidex, Middleton, WI), and zymosan (ICN) and were prepared, stored, and delivered as described previously (4). At the end of the specified incubation time the cells were washed three times with PD, and scraped into PD containing 1% (wt/vol) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin at 0.28 trypsin inhibitor unit/ml, 1 mM diisopropylfluorophosphate, and 15 mM EDTA (Sigma) (lysis buffer). Nuclei were removed by centrifugation for 5 min in an Eppendorf microcentrifuge and the protein content of the postnuclear supernatants was determined according to the method of Lowry *et al.* (16). Samples containing equiv-

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Abbreviations: 20:4, arachidonic acid; PMA, phorbol 12-myristate 13-acetate; LPS, bacterial lipopolysaccharide; MAGP, monoacyl glucosamine phosphate.

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alent amounts of protein were subjected to $NaDodSO_4$/PAGE on a 6–12% acrylamide gel according to Laemmli (17). Proteins were fixed/stained and prepared for fluorography by treatment with EN^{3}HANCE (New England Nuclear).

Determination of Hydroxylamine Sensitivity. The proteins of duplicate samples were separated by $NaDodSO_4$/PAGE. The gel was fixed in methanol/acetic acid, washed in water, and cut. Half the duplicate gel was agitated gently for 24 hr in 1 M hydroxylamine, pH 10, while the other half was handled similarly but in a solution of 1 M Tris, pH 7. The gels were then prepared for fluorography as described above. In agreement with previous reports (7), hydroxylamine treatment caused the release of radiolabel associated with proteins from cells labeled with [^{3}H]palmitic acid (data not shown).

Identification of the Protein-Bound Fatty Acid. The lipid species associated with protein was determined according to a modified version of the method of Olson *et al.* (7). Cells were labeled with [^{3}H]myristate in the presence of PMA and LPS as described above. The cell lysates were subjected to $NaDodSO_4$/PAGE and the regions of the gel containing the labeled protein species were identified by using prestained molecular weight standards (Bethesda Research Laboratories) that were run in adjacent lanes. The bands at 92, 68, 50, 42, 31, and 18 kDa were excised from the gel, homogenized in 0.1% $NaDodSO_4$/0.05 M NH_4HCO_3/5% (vol/vol) 2-mercaptoethanol, heated at 100°C for 5 min, and then incubated overnight with shaking in the presence of trypsin at 0.2 mg/ml. The gel extract was passed through a 0.22-μm Millex filter and the solution containing the tryptic fragments was acidified and extracted with petroleum ether to remove noncovalently bound fatty acids; no radioactivity was found in the organic phase. The aqueous phase was then lyophilized. The lyophilized material was suspended in 1 ml of 83% (vol/vol) methanol/2 M HCl, 10 μl each of 20 mg/ml stocks of myristic acid and palmitic acid (Sigma) were added, and the samples were then heated under reduced pressure at 110°C for 60 hr. The reaction solution was then extracted three times with 1 ml of petroleum ether. The combined petroleum ether extracts received 20 μl each of 20 mg/ml stocks of methyl myristate, methyl palmitate, myristic acid, and palmitic acid (Sigma) in HPLC-grade methanol. Samples were evaporated to dryness under nitrogen and redissolved in 250 μl of methanol. Separation and identification of the petroleum ether-extractable radioactivity were achieved by reverse-phase HPLC using a 4.6-mm × 25-cm Altex Ultrosphere ODS column eluted with 80% (vol/vol) acetonitrile/0.1% trifluoroacetic acid/0.02% triethylamine as the mobile phase at a flow rate of 1 ml/min. One-minute fractions were collected and their radioactivities were measured in Hydrofluor (National Diagnostics, Somerville, NJ). The absorbance elution profile of the methyl ester and free fatty acid internal standards were used to identify radioactive methanolysis products. A typical elution profile is shown in Fig. 3.

Subcellular Fractionation. To examine the distribution of acylated proteins between membrane and soluble fractions, macrophage cultures were labeled for 2 hr with [^{3}H]myristate as described above. The cells were then scraped into a fractionation buffer containing 0.25 M sucrose, 0.1 M $MgCl_2$, 10 mM Tris·HCl at pH 7.4, and the protease inhibitors included in the lysis buffer to a cell density of approximately 5×10^7 per ml. Cell suspensions were sonicated with three 5-sec bursts of 90 W each. The homogenate was centrifuged at 1000 × *g* for 15 min to remove unbroken cells and nuclei. The supernatant was then centrifuged at 100,000 × *g* for 30 min in a Beckman Airfuge to separate the cytosol and membrane fractions. The cytosolic and crude membrane fractions yielded approximately equivalent amounts of protein. Equivalent amounts of protein from soluble and membrane fractions were analyzed by electrophoresis on $NaDodSO_4$/polyacrylamide gels, followed by fluorography.

LPS Determination. All reagents and culture material were found to contain less than 0.1 ng of contaminating LPS per ml by the *Limulus* amebocyte lysate assay (Cape Cod Associates, Woods Hole, MA).

RESULTS

Myristoylation of Specific Proteins After Macrophage Activation. We have investigated protein acylation in murine peritoneal macrophages and have found that macrophages readily incorporate exogenous [^{3}H]myristic acid in culture. The vast majority (approximately 98%, data not shown) of the [^{3}H]myristic acid was incorporated into the phospholipid pool and ran with the dye front of $NaDodSO_4$/PAGE (Fig. 1). However, a detectable portion of the [^{3}H]myristic acid was specifically incorporated into characteristic proteins (bold arrows in Fig. 1). Thus, when macrophages were cultured for 2 hr in media containing 35 μCi of [^{3}H]myristic acid and the cell lysates were analyzed by $NaDodSO_4$/PAGE and autoradiography, a basal myristoylation of several proteins, particularly an 18-kDa species, was observed (Fig. 1, lane E). Several additional bands—e.g., 68 kDa and a doublet at 42 kDa—were present in various amounts in some experiments, a finding that may reflect the presence of contaminating LPS as discussed below.

Cells cultured in the presence of LPS or its monoacyl derivative MAGP for 2 hr exhibited specific myristoylation of a 68-kDa species, and a doublet of about 42 kDa (Fig. 1, lanes B and C) but did not affect the extent of myristoylation of the 14-kDa or 18-kDa proteins compared to that seen in unstimulated cells. Treatment of the cells with zymosan (Fig. 1, lane A) or PMA (Fig. 1, lane D) resulted in a labeled doublet at 42 kDa and a protein at 68 kDa that comigrated in $NaDodSO_4$/PAGE with the predominant myristoylated proteins induced by LPS. In addition, PMA or zymosan induced the myristoylation of a unique protein of 50 kDa and, to a lesser extent, a 92-kDa protein. The constitutively myristoylated

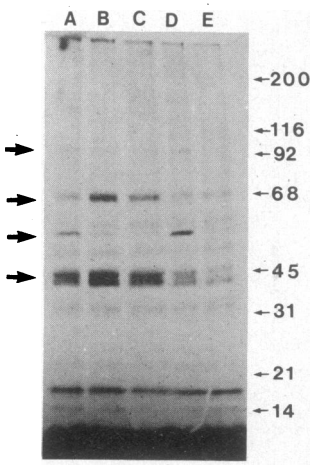

FIG. 1. Stimulus-dependent incorporation of [^{3}H]myristic acid into macrophage proteins. Resident peritoneal macrophages were isolated, plated at approximately 4×10^6 macrophages per 35-mm culture dish, and incubated overnight in α-MEM containing 10% fetal calf serum. The cells were then washed twice in PD and incubated in 1 ml of α-MEM containing 30 μCi of [^{3}H]myristic acid and the following stimuli: lane A, zymosan at 160 μg/ml; lane B, MAGP at 1 μg/ml; lane C, LPS at 1 μg/ml; lane D, PMA at 50 ng/ml; and lane E, no stimulus. After 2 hr at 37°C, the cells were scraped into lysis buffer and the postnuclear supernatant was analyzed by $NaDodSO_4$/PAGE on a 6–12% acrylamide gel. The fluorograph shown was exposed for 14 days. Numbers on the right are masses of marker proteins in kDa. Bold arrows indicate proteins of interest.

proteins—e.g., the 14-kDa and 18-kDa species, were unaffected by PMA or zymosan.

The distinct array of myristoylated proteins seen with LPS as compared to zymosan and PMA was particularly intriguing since we have previously shown that LPS is capable only of *priming* macrophages for enhanced 20:4 metabolism, whereas PMA or zymosan *triggers* 20:4 release (4).

Characterization of the Acylation Reactions. It has previously been shown that the myristoylation of proteins invariably occurs via a hydroxylamine-resistant amide linkage (7, 8), while palmitic acid is typically attached to proteins via hydroxylamine-sensitive ester bonds (7–9). The acylated proteins described above were found to be resistant to treatment with 1 M hydroxylamine, pH 10 (Fig. 2), suggesting that the fatty acid moiety was in an amide linkage. The identity of the fatty acid as myristic acid was confirmed by acid methanolysis of the proteins eluted from each band and identification of the methyl ester of the fatty acids by HPLC (Fig. 3). For the 92-, 68-, 50-, 42-, 31-, and 18-kDa proteins >98% of the radioactivity eluted with myristate plus methyl myristate and ≈1% as palmitate plus methyl palmitate.

To determine the fatty acid specificity of the acylation reactions the cells were cultured in the presence of the saturated fatty acid, [^{3}H]palmitic acid, which is readily incorporated into the cellular phospholipid pool (data not shown). Several labeled proteins were detected in the cell lysates of untreated and LPS-, zymosan-, or PMA-treated macrophages cultured in the presence of [^{3}H]palmitic acid (data not shown). The acyl linkage in this case, however, was hydroxylamine sensitive, and these labeled proteins did not comigrate with any of the myristoylated proteins upon NaDodSO$_4$/PAGE (data not shown). In contrast, no labeled proteins were detected when untreated or stimulated cells were cultured in media containing ^{3}H-labeled 20:4 (data not shown).

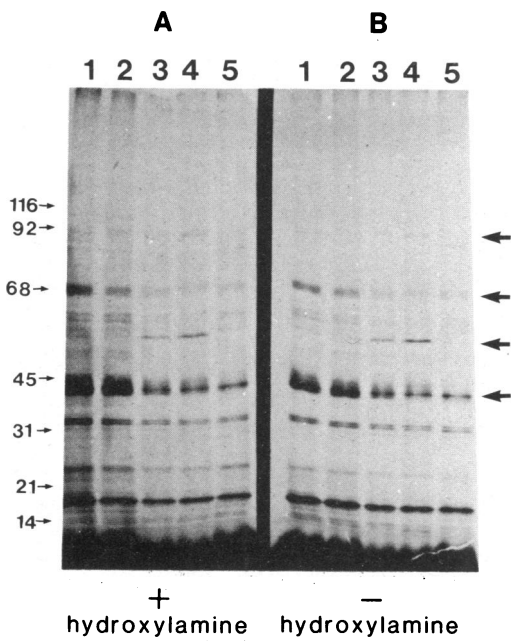

FIG. 2. Sensitivity of acylated proteins to hydroxylamine. Resident peritoneal macrophages were isolated and incubated with [^{3}H]myristic acid and various stimuli as described in the legend to Fig. 1. Lane 1, MAGP at 1 μg/ml; lane 2, LPS at 1 μg/ml; lane 3, zymosan at 160 μg/ml; lane 4, PMA at 50 ng/ml; lane 5, no stimulus. Duplicate samples were then subjected to NaDodSO$_4$/PAGE. After electrophoresis, fixing, and destaining the gel was cut in half. One half (*A*) was treated with 1 M hydroxylamine, pH 10, and the other (*B*), with 1 M Tris·HCl, pH 7, for 24 hr at room temperature with gentle agitation. Gels were then fixed, treated for fluorography, and exposed for 12 days.

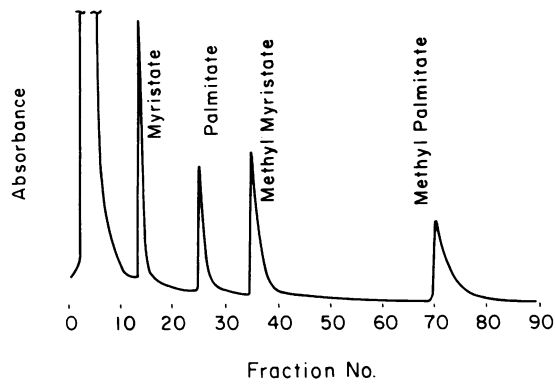

FIG. 3. HPLC elution profile of a set of fatty acid standards recorded at 214 nm.

Kinetics of Protein Myristoylation in Macrophages. The temporal response of protein acylation induced by LPS or PMA is shown in Fig. 4. Myristoylated 14- and 18-kDa proteins were detectable by 20 min and were maximally labeled by 45 min. The myristoylation of the 42-kDa proteins induced by both PMA and LPS was detected by 30 min. This was followed by the appearance of the 68-kDa myristoylated protein which was maximally labeled by 45 min in both cases. Finally, the labeling of the 50-kDa protein myristoylated uniquely in response to PMA (and zymosan) was apparent at 90 min (Fig. 4*B*). The heavily labeled acylated protein of 48 kDa seen in Fig. 4*B* is generally detected as a relatively minor band (see Fig. 1). Occasionally, this band is more heavily labeled, and preliminary results suggest that the "activation state" of the macrophage might influence the myristoylation of this protein. A complete study of this phenomenon will be reported elsewhere.

Prelabeling of the cells with [^{3}H]myristic acid did not have a significant effect on the time course of acylation of the myristoylated proteins, suggesting that the temporal response in labeling reflects the acylation reaction and is not due to differential equilibration of the label.

Subcellular Localization of the Myristoylated Proteins. The subcellular localization of the myristoylated proteins was determined by separating the cytosolic and membrane fractions. Cells were labeled with [^{3}H]myristic acid in the presence or absence of PMA and MAGP as described above. The cells were then disrupted, separated into membrane and cytosolic fractions as described in *Materials and Methods*, and analyzed by NaDodSO$_4$/PAGE and autoradiography. The results as depicted in Fig. 5 indicate that the majority of the induced myristoylated proteins were associated with the membrane fraction in both unstimulated and PMA- or MAGP-stimulated cells. The myristoylated proteins seen in the membrane fraction of MAGP-treated cells in the range 27–35 kDa, which are not apparent in Figs. 1 and 2, are seen variably from experiment to experiment.

Absence of LPS-Induced Myristoylation in Peritoneal Macrophages from C3H/HeJ Mice. Macrophages of C3H/HeJ mice are LPS nonresponders (18). We therefore compared LPS-induced protein myristoylation of macrophages from C3H/HeJ mice to that of macrophages from ICR mice (Fig. 6). The 14- and 18-kDa proteins were similarly myristoylated in macrophages from C3H/HeJ and control mice, indicating that the cells were equally capable of incorporating the fatty acid into protein. In marked contrast, none of the proteins specifically myristoylated in response to LPS in cells from normal mice were detectable in lysates of LPS-stimulated macrophages from C3H/HeJ mice (Fig. 6).

Interestingly, while PMA and zymosan promoted the myristoylation of the 50-kDa protein in C3H/HeJ mice, they did not induce the acylation of the 42-kDa doublet or the 68-kDa species (Fig. 6).

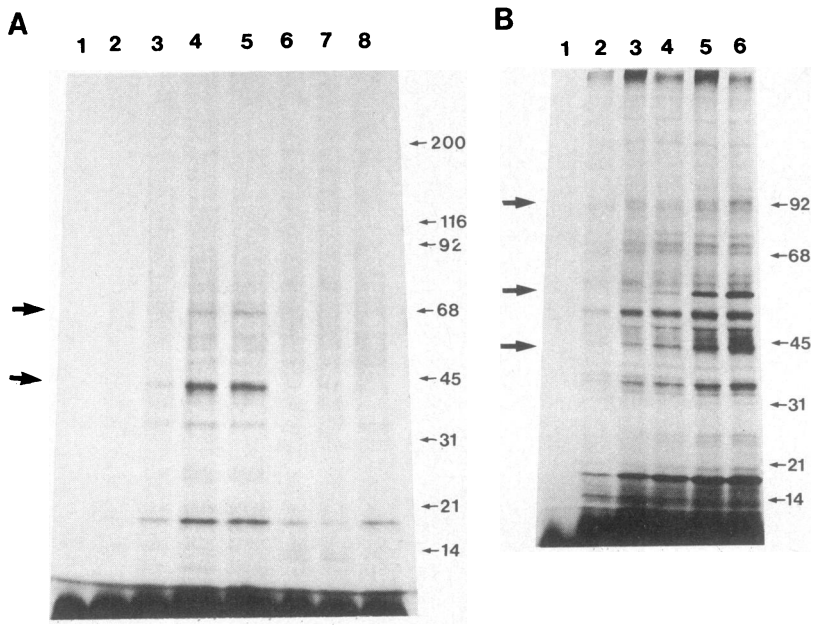

FIG. 4. Temporal sequence of myristoylation of macrophage proteins. Cells were incubated with [^{3}H]myristic acid alone (*A*, lanes 6–8), or with LPS at 1 μg/ml (*A*, lanes 1–5) or PMA at 50 ng/ml (*B*, lanes 1–6) for the following times. (*A*) Lane 1, 5 min; lanes 2 and 6, 20 min; lanes 3 and 7, 45 min; lane 4, 60 min; lanes 5 and 8, 90 min. (*B*) Lane 1, 5 min; lane 2, 20 min; lane 3, 45 min; lane 4, 60 min; lane 5, 90 min; lane 6, 120 min. The cells were then scraped in lysis buffer and the acylated proteins were identified by NaDodSO$_4$/PAGE and fluorography as described for Fig. 1.

DISCUSSION

We have shown the stimulus-dependent myristoylation of at least five specific proteins in primary macrophage cultures. Interestingly, the profile of protein acylation varies with the stimulus. Thus, LPS or its monoacyl derivative MAGP induces the specific labeling of three protein species, 68 kDa and a doublet at 42 kDa in NaDodSO$_4$/PAGE. Stimulation with zymosan or PMA results in the acylation of proteins that comigrate with each of those labeled in LPS-treated cells. However, the major species labeled in zymosan- or PMA-treated cells is a 50-kDa protein. With each labeled protein, the acyl moiety was linked to protein via hydroxylamine-resistant amide bonds. These acylation reactions are specific for myristic acid, since labeled proteins that comigrated with those described above were not detected in macrophages stimulated with LPS, zymosan, or PMA in the presence of ^{3}H-labeled palmitic acid or 20:4.

Recently a number of proteins involved in cellular regulation and transformation have been shown to undergo myristoylation. These include the catalytic subunit of the cAMP-dependent protein kinase and the calcium-binding protein calcineurin (10, 11). Both of these proteins play a pivotal role in cellular regulation. Furthermore, the pp60^{src} oncogene product of the Rous sarcoma virus and its 36-kDa substrate can be myristoylated (13, 19). The myristoylation of the pp60^{src} is necessary for virus-induced transformation. It

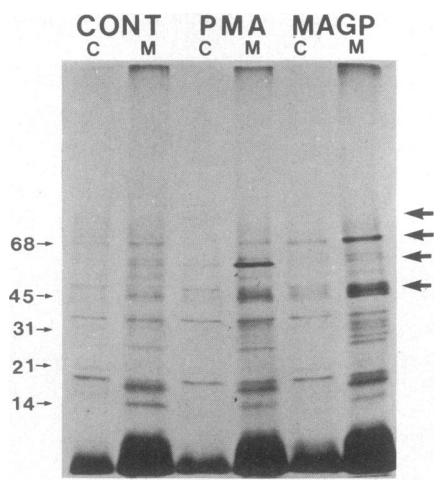

FIG. 5. Subcellular distribution of myristoylated proteins. Resident peritoneal macrophages were incubated with [^{3}H]myristic acid alone (CONT, control) or with PMA at 50 ng/ml or MAGP at 1 μg/ml. After 2 hr the cells were harvested and separated into membrane (M) and cytosolic (C) fractions. Equivalent quantities of protein from membrane and cytosolic fractions were then analyzed by NaDodSO$_4$/PAGE and fluorography.

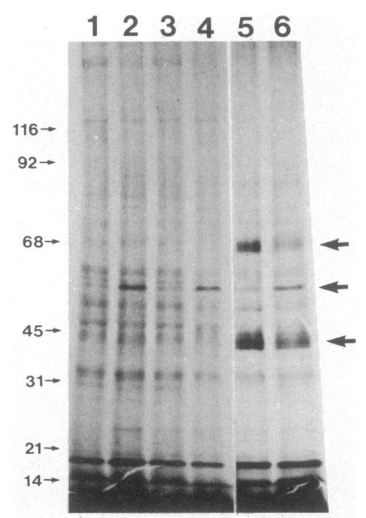

FIG. 6. Stimulus-dependent myristoylation of proteins in macrophages from C3H/HeJ mice. Resident peritoneal macrophages were harvested from C3H/HeJ mice (lanes 1–4) or ICR mice (lanes 5 and 6) and incubated as described for Fig. 1 with [^{3}H]myristic acid and the following stimuli: lane 1, no stimulus; lane 2, PMA at 50 ng/ml; lane 3, LPS at 1 μg/ml; lane 4, zymosan at 160 μg/ml; lane 5, LPS at 1 μg/ml; lane 6, PMA at 50 ng/ml. After 2 hr at 37°C, the cells were scraped into lysis buffer and the postnuclear supernatant was analyzed by NaDodSO$_4$/PAGE on a 6–12% acrylamide gel. The fluorograph shown was exposed for 14 days.

has been shown that the N-terminal 14 amino acids of $pp60^{src}$ direct the myristoylation and promote its association with the membrane (13). Furthermore, the p56 tyrosine kinase found in the LSTRA transformed cell line can exist as a myristoylated protein (12).

We have yet to identify or define the function of any of the myristoylated macrophage proteins. One of the acylated proteins found in the doublet at 42 kDa has a molecular mass similar to that of the catalytic subunit of the cAMP-dependent protein kinase that has previously been shown to be myristoylated (10). PMA is known to activate protein kinase C (approximately 90 kDa) (20), and it is tempting to speculate that the PMA-induced 90-kDa myristoylated protein might be protein kinase C. It has also been shown that protein kinase C can translocate from the cytosol to the plasma membrane upon cellular activation (21). Myristoylation of protein kinase C might promote this membrane attachment.

The differences observed in the acylation reactions induced by LPS vs. zymosan or PMA are particularly intriguing since we have recently shown that these stimuli differ in their capacity to influence 20:4 metabolism in macrophages (4). Thus, PMA and zymosan cause the secretion of 20:4 metabolites. In contrast, LPS alone results in release of low levels of 20:4 metabolites but is effective in priming these cells for enhanced 20:4 secretion in response to subsequent stimulation with zymosan or PMA. Furthermore, investigation of the kinetics of 20:4 release demonstrates that pretreatment of cells with LPS eliminates that lag phase of the response seen when cells are stimulated with zymosan or PMA alone. These data led us to suggest that triggers of 20:4 metabolism can induce two sequential signals: a priming signal and a triggering signal. LPS appears to provide only the priming signal to macrophages, while zymosan or PMA could provide both signals. It is possible that the acylation of distinct proteins induced by LPS vs. zymosan or PMA reflects the difference in the capacity of these stimuli to prime vs. trigger macrophage 20:4 metabolism. This hypothesis is supported by the fact that the appearance of the myristoylated proteins that are common in the LPS-, zymosan-, and PMA-induced cells parallels the kinetics of macrophage priming by LPS (Fig. 4 and ref. 4). The acylation of the 50-kDa protein that is unique to macrophages triggered to release 20:4 metabolites—e.g., PMA—is delayed compared to the other acylated proteins. Taken together, these data lead us to speculate that the myristoylation of the 42- and 68-kDa proteins is involved in the priming signal, while the myristoylation of the 50-kDa protein might be an integral part of the secretory process. It is further possible that the covalent transfer of the myristoyl moiety of LPS to macrophage proteins might be the molecular basis of priming.

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