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S L Bursten, … , J L Ryan, D H Lovett

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Research Article

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Acylation of Monocyte and Glomerular Mesangial Cell Proteins

Myristyl Acylation of the Interleukin 1 Precursors

Stuart L. Bursten,* Richard M. Locksley,‡ John L. Ryan,§ and David H. Lovett‖

**Medical Service, Seattle Veterans Administration Medical Center-University of Washington, Seattle, Washington 98108; ‡Department of Medicine, University of California at San Francisco, California 94143; §Medical Service, West Haven Veterans Administration Medical Center-Yale University, New Haven, Connecticut 06516; ‖University of California Medical Service, San Francisco Veterans Administration Medical Center, San Francisco, California 94121*

Abstract

Acylation of cellular proteins with the fatty acids myristate or palmitate represents an important mechanism for the co- or posttranslational modification of proteins. Lipid A, the biologically active component of bacterial endotoxin, exerts a number of biochemical effects on responsive cell types. Evidence is presented that lipid A stimulates the synthesis and subsequent myristyl acylation of intracellular monocyte and glomerular mesangial cell proteins. Two of the myristylated monocyte proteins were identified by specific immunoprecipitation as the 33-kD IL 1 α and β precursors; a similar myristylated protein was found in mesangial cells. The 17-kD secretory form of monocyte IL 1 β did not contain covalently linked myristate. Myristyl acylation of the IL 1 precursor proteins may facilitate the processing or membrane localization of these proteins, which lack characteristic hydrophobic signal sequences. The acylated 33-kD IL 1 α may remain preferentially associated with the membrane in an active form, whereas limited proteolysis may convert the biologically inactive IL 1 β precursor into the extracellular, nonacylated, active 17-kD protein.

Introduction

The activation of eukaryotic cells by the gram-negative bacterial cell wall component, lipopolysaccharide (LPS), has been extensively documented. Until recently, little was known about the precise biochemical mechanisms whereby LPS, or its biologically active component, lipid A, elicited the diverse group of responses included under the term cellular activation. Studies using murine peritoneal macrophages have shown that one early action of LPS is the induction of a set of new genes, including the c-*myc* and c-*fos* protooncogenes (1). These events are associated with the enhanced synthesis of several specific macrophage proteins that may play a role in modulating the cellular response to LPS (2, 3). Alterations in the pattern of protein phosphorylation and hydrolysis of polyphosphoinositides have also been attributed to LPS action (4). Although significant, these investigations have focused upon biochemical alterations in murine peritoneal macrophages and it remains to be determined whether such events represent LPS activation of all responsive cell types.

Address correspondence to Dr. David H. Lovett, 111J Medical Service, San Francisco Veterans Administration Center, 4150 Clement St., San Francisco, CA 94121.

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Recent studies from this laboratory (5, 6) indicate that glomerular mesangial cells (MC)[1] are LPS responsive. Activation of glomerular MC by bacterial cell wall components rapidly alters cellular morphology and stimulates the release of prostanoids and protein inflammatory mediators, including IL 1 (5, 6). These mediators may play an important role in the alterations in glomerular hemodynamics characteristic of septicemia and in the induction of the cell proliferation that accompanies many types of postinfectious glomerulonephritis.

In addition to the biochemical alterations discussed above, LPS was recently found to stimulate the acylation of several proteins in resident murine peritoneal macrophages (7). These findings suggested that LPS-dependent acylation of newly synthesized proteins may represent an additional mechanism whereby endotoxins modulate the cellular activation process. Protein fatty acid acylation involves primarily the covalent binding of palmitate or myristate through the action of specific acyltransferase enzymes. The posttranslational, thioester linkage of palmitate has been demonstrated for several critical cellular proteins, including the transferrin receptor and the HLA glycoprotein (8–10). In contrast, the myristyl acylation of proteins occurs through amide bonds as a cotranslational event (11, 12). Several myristylated cellular proteins have been identified, including the catalytic subunit of cAMP-dependent protein kinase, $p60^{src}$, the p56 tyrosine kinase, and various *gag-onc* fusion proteins (13–19). The coupling of long-chain fatty acids to proteins alters a number of functional properties in addition to facilitating membrane attachment, including transformation potential, subunit interaction, and intermolecular packing (13, 15, 20–24).

Given these observations and the potential regulatory significance of LPS-dependent protein acylation, experiments were performed to examine the effects of LPS on the rates and patterns of protein acylation by a nonmacrophage cell type, the glomerular MC, and to compare these events with a classical LPS-responsive cell, the monocyte. Evidence is presented that lipid A, the biologically active component of LPS, induces the synthesis and myristyl acylation of a limited number of mesangial cell and monocyte proteins. Two prominent lipid A-induced, 33-kD myristylated proteins were identified as the IL 1 precursor molecules, a finding that may be related to the complex cellular processing of these critical inflammatory mediators.

Methods

Reagents. ITS (insulin, transferrin, and selenous acid), containing 5 μg/ml transferrin, 1×10^{-6} M insulin, and 5 ng/ml selenous acid (final

1. *Abbreviations used in this paper:* IEF, isoelectric focusing; ITS, insulin/transferrin/selenous acid; MC, mesangial cells.

concentrations in medium), was obtained from Collaborative Research (Waltham, MA). Lipid A, purified from the *Staphylococcus minnesota* R595 strain, was obtained from Ribi Immunochem Research, Inc. (Hamilton, MT). The lipid A was prepared as a stock solution of 1 μg/ml in RPMI 1640 supplemented with 0.1% defatted BSA. Immediately before use, the stock solution of lipid A was briefly sonicated on ice. Pansorbin (fixed protein A-bearing *Staphylococcus aureus*) for radioimmunoprecipitation was obtained from Calbiochem-Behring Corp., La Jolla, CA. Media and heat-inactivated fetal bovine serum (FBS) were obtained from Gibco Laboratories (Grand Island, NY). [^{35}S]Methionine (1,350 Ci/mmol), and [^{3}H]myristate (22 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Before use, the media components were absorbed with polymixin B-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and were free of detectable endotoxin contamination (< 0.1 ng/ml) when assessed by the *Limulus* amoebocyte lysate assay. Purified human recombinant 33-kD IL 1 β precursor protein, rabbit polyclonal anti-human recombinant II 1 β IgG and murine (IgG_1) monoclonal anti-human IL 1 α (25) were the generous gifts of R. Black and K. Grabstein, Immunex Corp., Seattle, WA. The MAb was prepared against a synthetic peptide corresponding to a unique sequence of amino acid residues (256–271) of IL 1 α. A second rabbit polyclonal anti-human IL 1 IgG preparation was obtained from Endogen, Boston, MA. This antibody was prepared against purified natural human IL 1, and recognizes common epitopes on the secreted 17-kD forms of both IL 1 α and IL 1 β (confirmed by Western blot analysis in our laboratory).

Preparation of cells. MC growth medium consisted of DME supplemented with 20% FCS, ITS, 300 μg/ml glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The culture of rat MC has been described in detail in previous reports (26, 27). In brief, primary cultures were obtained as outgrowths from collagenase-treated glomerular remnants derived from blood-free, perfused kidneys obtained from 150-g male Sprague-Dawley rats. MC appeared as outgrowths within 5–7 d and small clusters of cells with typical morphology were picked, replated, and grown to confluence. The cultures were subsequently rapidly expanded and uniform populations were frozen in liquid nitrogen at the fourth to sixth passages. Using previously reported criteria (26–28), the cells were characterized as intrinsic MC. Subconfluent MC layers were washed three times with warm PBS before stimulation studies (see below).

Human monocytes were obtained from normal donors using standard methodology. In brief, heparinized blood was diluted 1:1 with PBS, pH 7.2, before separation over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) after centrifugation for 15 min at 2,000 *g*. The mononuclear cell interface was collected, washed three times in calcium-free HBSS, and distributed to 100-mm plastic dishes at a concentration of 5×10^6 cells/ml in RPMI 1640 containing 5% FBS. After incubation at 37°C in 5% CO_2 for 2 h, nonadherent cells were removed by vigorous washing. The adherent monolayers consisted of > 95% monocytes as assessed by nonspecific esterase staining and phagocytosis of zymosan. The adherence-purified monocytes were cultured for 24 h before lipid A stimulation.

Experimental protocol. For labeling with [^{35}S]methionine, washed layers of MC or monocytes were incubated for 60 min in methionine-deficient DME. The medium was removed and replaced with fresh methionine-deficient medium containing 100 μCi/ml [^{35}S]-methionine. Experimental groups were stimulated for varying time periods with 100 ng/ml lipid A. At the appropriate times, the culture supernatants were removed, centrifuged at 400 *g* for 10 min, supplemented with protease inhibitors (5 mM EDTA, 0.2 mM PMSF, 2 μM pepstatin), and frozen at −70°C. Cell layers were washed twice with cold PBS, scraped into microfuge tubes, and centrifuged at 10,000 *g* for 5 min. The cell pellets were washed an additional two times with cold PBS before preparation for electrophoretic analysis or immune precipitation (see below).

For labeling with [^{3}H]myristate, the fatty acid was dried under nitrogen and brought into solution at 25 μCi/ml by gentle sonication in medium supplemented with 0.1% defatted BSA. After incubation with lipid A, the cell supernatants and layers were processed as detailed below.

The amount of cellular protein was quantitated according to Bradford (29), using BSA as a standard, and the specific activities of the proteins were determined by liquid scintillation counting. Specific activities are expressed as means of triplicate determinations.

Immune precipitation. Cell pellets from [^{35}S]methionine- or [^{3}H]-myristate-labeled human monocytes were lysed in an equal volume of 2× lysis buffer consisting of (final concentrations) 2% NP-40, 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.2, 0.68 M sucrose, and protease inhibitors as noted above. The lysates were incubated on ice for 30 min, sonicated for three 10-s bursts on ice at a power setting of five (Bronson Ultrasonics, Danbury, CT), and centrifuged at 10,000 *g* for 15 min. For immune precipitation of radiolabeled intracellular IL 1, the cell lysates were precleared by incubation at 4°C overnight with 10% (vol/vol) Pansorbin. The cleared lysates were then incubated for 18 h at 4°C with specific anti-IL 1 antibodies. For IL 1 β, the lysates were incubated with a 1:500 dilution of polyclonal rabbit anti-recombinant-IL 1 β IgG. IL 1 antigen-antibody binding was competed by the addition of purified nonradiolabeled 33-kD recombinant IL 1 β precursor in concentrations ranging from 0 to 1 μg/ml. Monoclonal anti-IL 1 α was used at a 1:1,000 final dilution. Nonimmune rabbit IgG was used as a negative control. After incubation, 10% Pansorbin was added and the bound immune complexes were recovered by centrifugation. The pelleted bacteria were washed five times in large volumes of wash buffer containing 0.5% NP-40, 0.45 M NaCl, and 0.05 M Tris/HCl, pH 8.3. The pellets were finally resuspended in isoelectric focusing (IEF) sample buffer (9.3 M urea, 5 mM K_2CO_3, 2% NP-40, 2% 3–10 ampholines, and 30 mM DTT). After a 2-h incubation at 4°C, the samples were centrifuged and analyzed by two-dimensional electrophoresis (see below). Immune precipitation of biosynthetically labeled IL 1 from the monocyte culture supernatants was performed in an identical fashion using the rabbit anti-IL 1 IgG (5 μg/ml) that recognizes epitopes on the 17-kD forms of both IL 1 α and IL 1 β. Nonimmune rabbit IgG was used as a negative control.

Electrophoretic methods. Labeled proteins were analyzed by both one- and two-dimensional electrophoresis. For these studies, equal amounts of either protein or radioactivity (1×10^6 dpm) were analyzed. SDS-PAGE was performed on 12.5% discontinuous gels according to Laemmli (30). For two-dimensional analysis, the labeled proteins were separated by IEF on 0.4-mm ultrathin polyacrylamide gels according to Goldsmith et al. (31) using 1.4% 4–6 ampholines and 0.6% 3–10 ampholines (LKB Instruments, Inc., Bromma, Sweden). Focused proteins were separated in the second dimension on 12.5% discontinous gels precisely according to O'Farrell (32). After electrophoresis, the gels were fixed in 10% TCA/30% ethanol for 45 min, washed twice in 5% TCA/30% ethanol, and soaked in En^{3}Hance (New England Nuclear). The gels were dried under heat and vacuum and analyzed by autoradiography at −70°C using preflashed Kodak X-O-Mat film with intensifying screens. The films were exposed under linear conditions. Analysis of the autoradiograms was performed by linear densitometry and computer imaging analysis using an X-Y digitizer (Bioquant, Nashville, TN). Isoelectric points were determined from the migration patterns of stained standard proteins (IEF calibration kit; Pharmacia Fine Chemicals). Phosphorylase b (94 kD), BSA (67 kD), ovalbumin (45 kD), trypsin inhibitor (20 kD), and lactalbumin (14 kD) were used as molecular weight standards for SDS-PAGE.

Immunoblotting. Secreted IL 1 from the supernatants of human monocytes incubated with [^{3}H]myristate and lipid A was immune precipitated as detailed above. The immune precipitates were solubilized in reducing Laemmli sample buffer and electrophoresed on 12.5% discontinuous gels. After electrophoresis, the proteins were transferred to nitrocellulose and blocked in PBS containing 50 g/liter nonfat dried milk and 0.5% Tween 20. Blocked sheets were washed in PBS/Tween and incubated for 18 h at 4°C with 5 μg/ml rabbit anti-human IL 1 IgG. Thereafter, the sheets were washed and incubated at 25°C with a 1:1,000 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 2 h. The sheets were

washed and developed with alkaline phosphatase-coupled biotin-avidin complexes (Vector Laboratories). Developed blots were photographed and sprayed with En^{3}Hance for autoradiography.

Analysis of acylated proteins. The cellular lysates from human monocytes incubated with [^{3}H]myristate and lipid A were separated by two-dimensional electrophoresis and transferred to nitrocellulose as detailed above. The proteins of interest were identified by autoradiography and cut out. Noncovalently bound fatty acids were removed by washing the nitrocellulose in chloroform/methanol (2:1) until the radiation approached background levels. Thereafter, acid methanolysis of the acylated proteins was performed by heating the samples at 110°C for 60 h in a solution of 83% methanol/2 M HCl, containing 10 μl each of 20-mg/ml stock solutions of myristic and palmitic acid (Sigma Chemical Co.). The reaction solution was extracted three times with petroleum ether and 20 μl each of 20 mg/ml stock solutions of methyl myristate and methyl palmitate were added. The samples were evaporated under nitrogen, resuspended in methanol and separated and identified by reverse-phase HPLC using a 4.6 mm × 25 cm C18 column (Rainin Instrument Co., Woburn, MA). The column was developed with 80% (vol/vol) acetonitrile/0.1% trifluoroacetic acid/0.5% triethylamine at a flow rate of 1 ml/min. Serial fractions were collected, suspended in counting scintillant (Amersham Corp., Arlington Heights, IL) and quantitated by liquid scintillation counting. The elution profile of radioactivity was compared with the absorbance elution profiles of standard palmitic and myristic acids and their respective methyl esters.

Results

In the first series of experiments, the short-term effects of lipid A on the rates of protein synthesis and acylation by human peripheral blood monocytes were examined. For these studies, the monocytes were incubated with lipid A in the presence of [^{35}S]methionine or [^{3}H]myristate and compared with non-stimulated controls. Preliminary studies had demonstrated that 100 ng/ml lipid A had a maximal effect on the rates of cellular protein acylation and this concentration was used without further modification. Stimulation of monocytes for 4 h with lipid A (Fig. 1) enhanced the synthesis of several cellular proteins, as determined by two-dimensional electrophoretic analysis. A closely migrating, [^{35}S]methionine-labeled protein doublet with a molecular mass of 33-kD and pI of ~ 7.1 and 7.3 was particularly prominent in the lipid A-treated cell lysates (*B*). The synthesis of the 33-kD protein doublet was increased over 10–20-fold under these conditions and represented the major lipid A-induced monocyte protein. The synthesis of a prominent 26-kD, 6.2-pI protein was also stimulated by lipid A, although to a lesser extent.

Incubation of human monocytes with [^{3}H]myristate for 4 h led to the acylation of a small number of cellular proteins (Fig. 2 *A*). A major increase in the number of acylated proteins was noted after stimulation with lipid A (*B*). Significantly, both components of the 33-kD protein doublet were myristyl acylated, a process that was greatly enhanced by lipid A.

The effects of lipid A on monocyte protein synthesis and acylation were detectable as early as 1 h after stimulation (Fig. 3). Control monocytes incubated with labeled myristate for 1 h demonstrated a limited amount of protein acylation, with most of the label incorporated into a protein with a molecular mass of 26 kD and pI of 6.2 (*A*). In contrast, stimulation for 1 h with 100 ng/ml lipid A enhanced the myristyl acylation of a number of monocyte proteins, with the acylation of the 33-kD protein doublet being particularly prominent (*B*). These acylated proteins represent newly synthesized monocyte proteins and match up precisely with the two-dimensional autoradio-

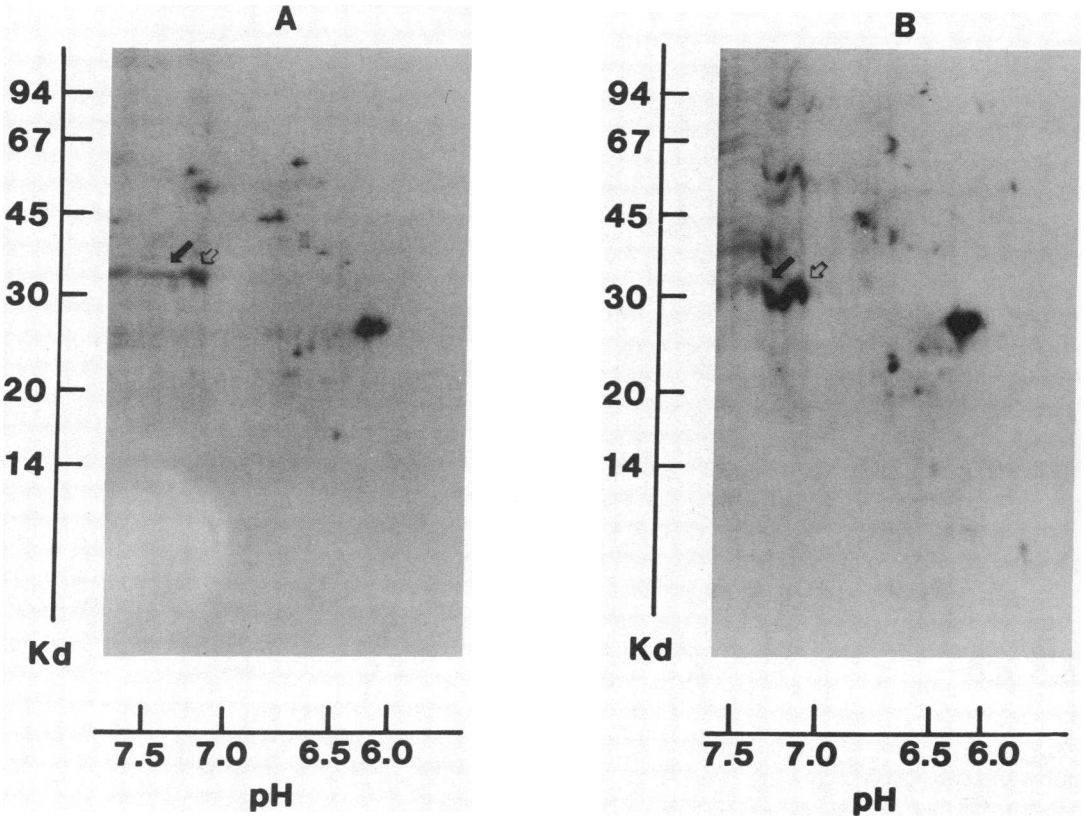

Figure 1. Two-dimensional electrophoretic analysis of lysates from human peripheral blood monocytes incubated with [^{35}S]methionine for 4 h. (*A*) Controls; (*B*) lipid A treated. *Arrows,* prominent 33-kD protein doublet, which consists of proteins with pI of 7.3 (*closed small arrow*) and 7.1 (*open small arrow*).

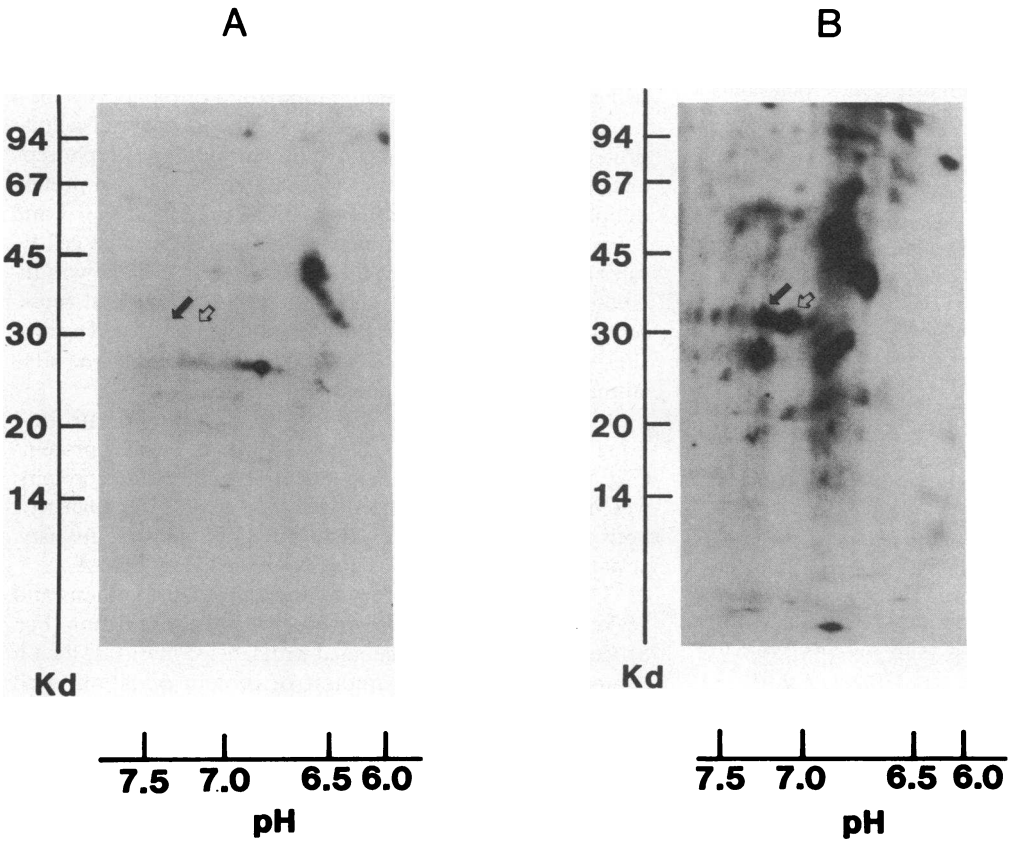

Figure 2. Two-dimensional electrophoretic analysis of lysates from human monocytes incubated with [^{3}H]myristate for 4 h. (*A*) Controls; (*B*) lipid A treated. The positions of the pI 7.3 (*closed arrows*) and pI 7.1 (*open arrows*) components of the 33-kD protein doublet are denoted.

gram from monocytes incubated with lipid A and [^{35}S]-methionine for 1 h (*C*). Further confirmation of the cotranslational nature of the myristyl acylation process was provided by studies using cycloheximide inhibition of protein synthesis, in which myristyl acylation was completely blocked (data not shown).

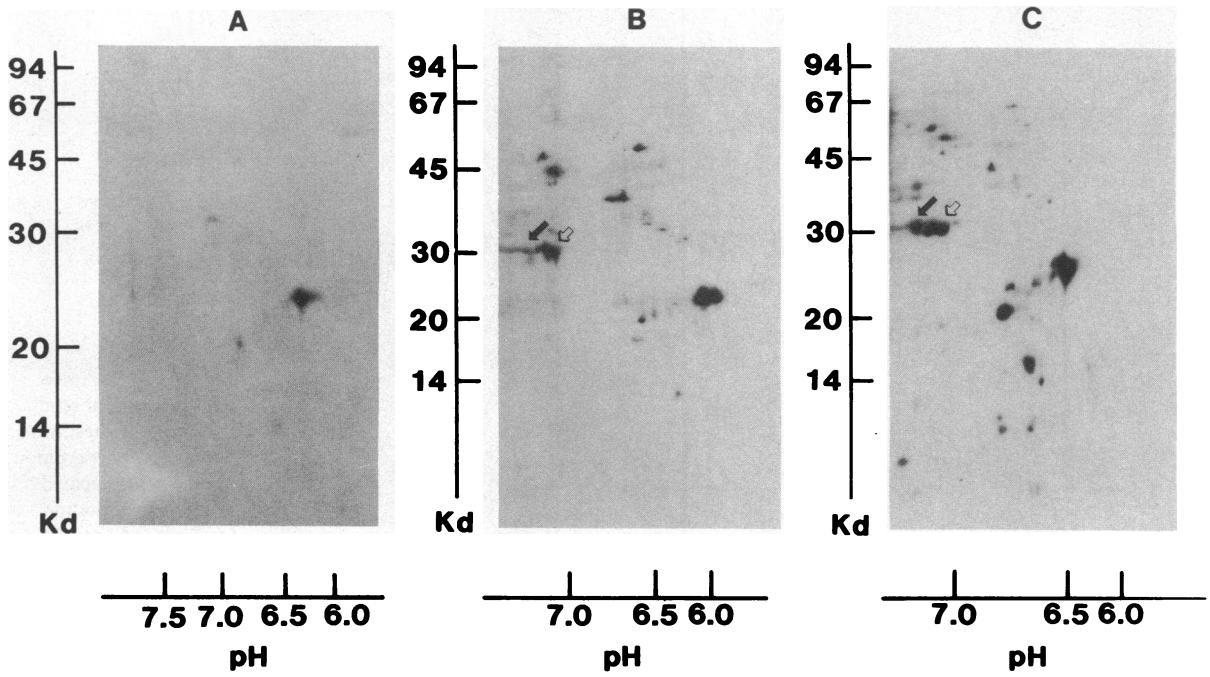

Figure 3. Analysis of lysates from human monocytes incubated with [^{3}H]myristate or [^{35}S]methionine for 1 h. (*A*) Control, myristate label; (*B*) lipid A treated, myristate label; (*C*) lipid A treated, methionine label. The arrows in *B* and *C* denote the pI 7.3 (*closed arrow*) and pI 7.1 (*open arrow*) components of the 33-kD doublet, whose synthesis and acylation are greatly enhanced after 1 h of lipid A stimulation.

Additional experiments were performed with glomerular MC to compare the initial observations concerning human monocytes with a second lipid A-responsive cell type. Stimulation of cultured MC for 4 h with lipid A enhanced the synthesis of a large number of proteins, as assessed by the incorporation of radiolabeled methionine (Fig. 4, Part I). Determination of specific activities indicated an approximate 2.5-fold stimulation of total MC protein synthesis by lipid A (controls: 4.7 $\times 10^4$ cpm/10 μg protein vs. lipid A: 11.3 $\times 10^4$ cpm/10 μg protein). A specific enhancement in the synthetic rates of a smaller number of mesangial proteins was also present when equal amounts of radioactivity were subjected to electrophoresis. The prominent protein at 33 kD (*arrow*) was specifically stimulated by a factor of two to three under these conditions. Myristyl acylation of several MC proteins was readily observed in control cultures incubated with the labeled fatty acid for 4 h (Fig. 4, Part II). Addition of lipid A to the cultures led to enhanced myristyl acylation of several of these proteins, the most prominent of which were 94, 60, 40, 33, and 28 kD in size, respectively. When equivalent amounts of radioactivity were electrophoresed, the myristyl acylation of the prominent 33-kD protein was found to be specifically stimulated by a factor of two to three. Two-dimensional electrophoretic analysis of the [^{35}S]methionine-labeled MC lysates provided further characterization of the prominent 33-kD protein, which contained two components with pI of 7.2 and 6.3 (Fig. 5 *A*). The synthesis of each component of the 33-kD protein was enhanced by incubation with lipid A (Fig. 5 *B*). Two-dimensional electrophoretic analysis of [^{3}H]myristate-labeled cellular lysates yielded an identical pattern (data not shown).

The nature of the prominent acylated 33-kD monocyte and MC proteins suggested a relationship to the intracellular precursors of IL 1 (33, 34). To specifically address this issue, immunoprecipitates from [^{35}S]methionine- and [^{3}H]-myristate-labeled monocytes were analyzed after 4 h stimulation with lipid A. Using a highly specific polyclonal anti-human recombinant IL 1 β IgG preparation, a single labeled protein was recovered from the lysates of monocytes incubated with [^{35}S]methionine and was found to have a molecular mass of 33 kD and pI of 7.3–7.4 (Fig. 6 *A*). Addition of increasing concentrations of purified recombinant 33-kD IL-1 β protein effectively competed for the precipitation of the labeled protein (Fig. 6 *B*). Immunoprecipitates from monocytes incubated with [^{3}H]myristate and lipid A yielded a labeled protein with the same molecular weight, isoelectric point, and pattern

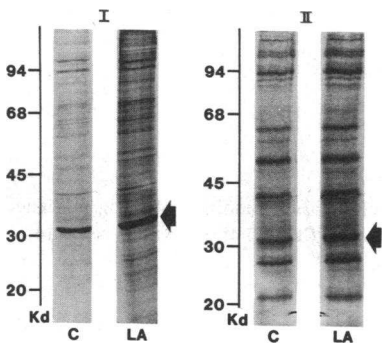

Figure 4. Effects of lipid A on protein synthesis and acylation by glomerular MC. MC were incubated for 4 h with: (I) [^{35}S]methionine, or (II) [^{3}H]myristate in the presence (LA) or absence (C) of 100 ng/ml lipid A. The cell layers were lysed, electrophoresed on 12.5% SDS-PAGE gels, and autoradiographed. *Thick arrows,* the prominent 33-kD protein.

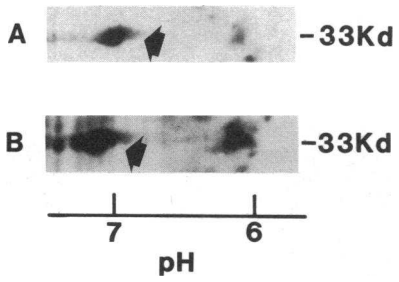

Figure 5. Two-dimensional electrophoretic analysis of the prominent 33-kD MC protein noted in Fig. 1. Cellular lysates from MC incubated for 4 h with [^{35}S]-methionine in the absence (*A*) or presence (*B*) of 100 ng/ml lipid A were separated by two-dimensional electrophoresis and autoradiographed. The 33-kD protein consists of two primary charge species, with pI of 7.2 (*arrow*) and 6.1–6.2. The synthesis of both proteins is significantly enhanced by incubation with lipid A.

of competition with excess recombinant IL 1 β (Fig. 7, *A* and *B*). These experiments establish that the newly synthesized, myristylated 33-kD, pI-7.3–7.4 monocyte protein is the monocyte IL 1 β precursor molecule. Using a specific MAb that had been prepared against a synthetic peptide corresponding to a unique amino acid sequence (256–271) of IL 1α (25), the 33-kD IL 1 α precursor was also immunoprecipitated (Fig. 8). A single [^{35}S]methionine- (Fig. 8 *A*) and [^{3}H]myristate- (Fig. 8 *B*) labeled protein with a molecular mass of 33 kD and pI of 7.1–7.2 was recovered using this specific MAb. The prominent myristylated protein doublet thus consists of the IL 1 β (pI 7.3–7.4) and the IL 1 α (pI 7.1–7.2) precursor molecules.

To confirm that the radiolabel present in the IL 1 precursor molecules represented intact, covalently linked myristic acid, acid methanolysis of the 33-kD, radiolabeled protein doublet was performed. After stimulation with lipid A for 4 h in the presence of [^{3}H]myristate, the monocyte lysates were separated by two-dimensional electrophoresis and transferred to nitrocellulose sheets. The IL 1 precursor proteins were identified by autoradiography, and washed in chloroform/methanol to remove noncovalently bound fatty acid. After acid methanolysis and extraction, the labeled fatty acids and methyl esters were separated by HPLC (Fig. 9). Approximately 85% of the radiolabel on the IL 1 precursor molecules was determined to be myristate (sum of labeled myristate and methyl myristate). A small amount (15%) of palmitate was also detected. The myristyl-labeled IL 1 proteins also were resistant to hydrolysis by hydroxylamine (data not shown), a finding consistent with amide rather than thioester covalent linkage of the fatty acid to protein (10, 11).

Further experiments were performed to determine if the extracellular, 17-kD form of monocyte IL 1 contained covalently linked myristate. These experiments used immunoprecipitation of monocyte supernatants with a specific anti-human IL 1 IgG that recognizes epitopes on both the 17-kD, pI-5.0 (α) and -7.0 (β) forms of extracellular IL 1. Electrophoretic analyses and autoradiography of the resultant immune complexes were performed. Monocytes were stimulated with lipid A for 6 h in the presence of either [^{35}S]methionine or [^{3}H]myristate, after which the supernatants were analyzed. A single 17-kD radiolabeled protein with pI of 7.3–7.0, consistent with its identification as IL 1β, was specifically immune precipitated from the supernatants of methionine-labeled monocytes (Fig. 10). Precipitation of the labeled 17-kD molecule could be blocked by competition with excess cold recombinant IL 1 β (data not shown). When the experiment was

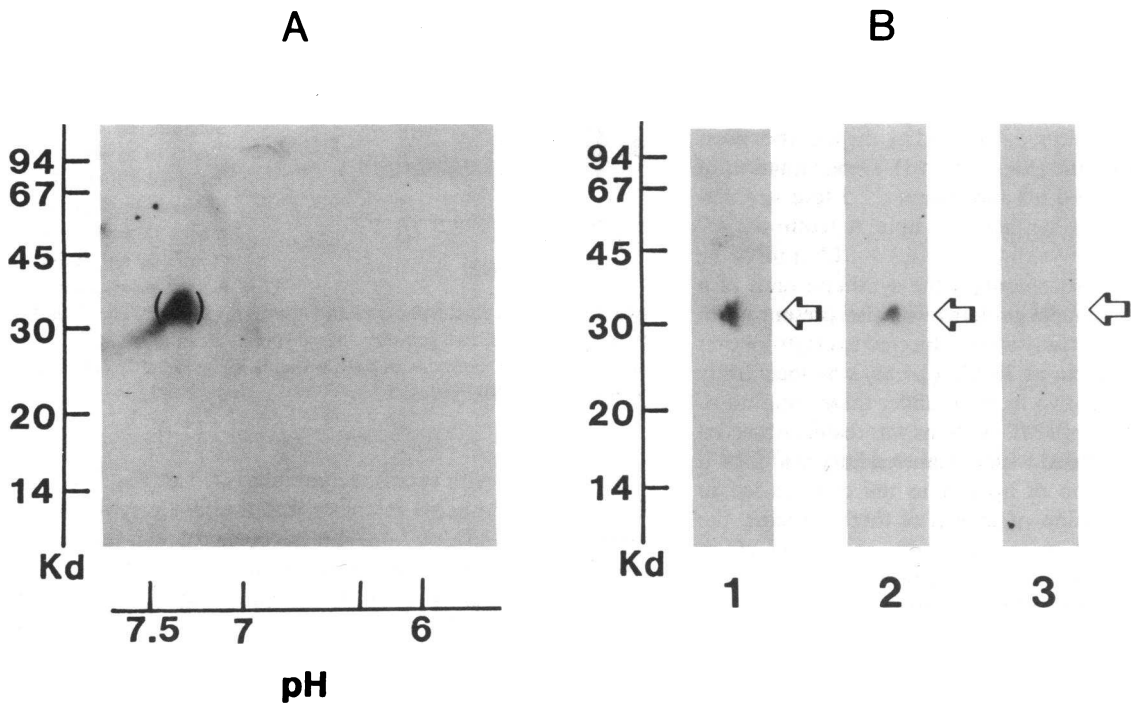

Figure 6. Immune precipitation of the 33-kD IL 1 β precursor from [^{35}S]methionine-labeled monocyte lysates, prepared as detailed in Methods. (*A*) Two-dimensional analysis, demonstrating precipitation of a 33-kD, pI 7.3–7.4, [^{35}S]methionine-labeled protein. (*B*) Competition of the [^{35}S]methionine-labeled IL 1 β precursor immune precipitation by addition of 10 ng/ml (lane *1*), 100 ng/ml (lane *2*), and 1 μg/ml (lane *3*) of nonlabeled purified recombinant IL 1 β precursor protein.

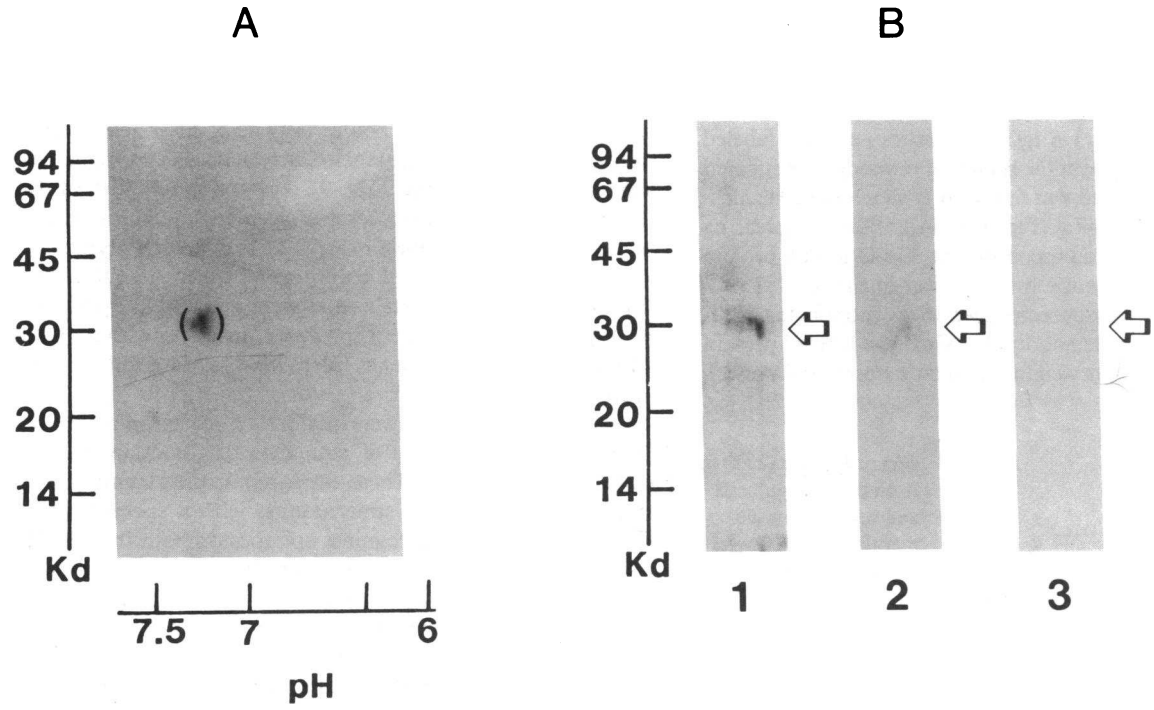

Figure 7. Immune precipitation of the 33-kD IL 1 β precursor from [^{3}H]myristate-labeled monocyte lysates, prepared as detailed in Methods. (*A*) Immunoprecipitation of a 33-kD, pI 7.3–7.4, [^{3}H]-myristate-labeled protein. (*B*) Competition of [^{3}H]myristate-labeled IL 1 β precursor immune precipitation by addition of 10 ng/ml (lane *1*), 100 ng/ml (lane *2*), and 1 μg/ml (lane *3*) of nonlabeled recombinant IL 1 β precursor protein.

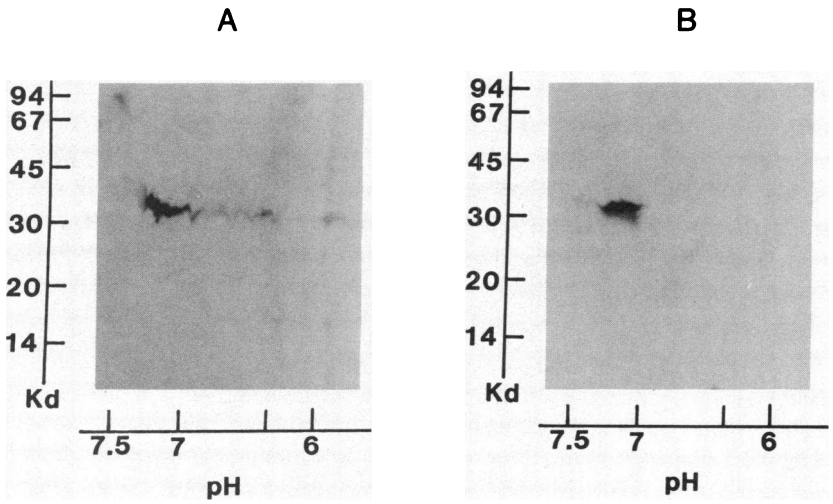

Figure 8. Immune precipitation of the 33-kD, pI 7.1–7.2, IL 1 α precursor protein using a specific monoclonal anti-IL 1 α antibody. (*A*) [^{35}S]-Methionine-labeled monocyte lysates. (*B*) [^{3}H]-Myristate-labeled monocyte lysates.

repeated under identical conditions using supernatants from myristate-labeled monocytes, there was no detectable [^{3}H]-myristate label present in the immunoprecipitates (Fig. 10, lane *A*). Exposure of the autoradiogram for up to 3 mo failed to detect [^{3}H]myristate radiolabel. A Western blot of the electrophoresed immune complexes confirmed that the 17-kD form of monocyte IL 1 β had been immune precipitated in these experiments (Fig. 10, lane *B*). Thus, lipid A stimulation of human monocytes led to the release of the 17-kD extracellular form of IL 1 β, which contained [^{35}S]methionine, but which did not contain detectable [^{3}H]myristate.

Discussion

These studies have demonstrated the general similarities of glomerular MC and human peripheral blood monocyte responses to short-term stimulation with biologically relevant concentrations of lipid A. Lipid A stimulation of each cell type for 4 h led to enhancements in the rates of overall protein synthesis and myristyl acylation. The actual magnitude of the monocyte synthetic responses to lipid A were much greater than those of MC. In both cell types, myristyl acylation of a limited number of proteins occurred, a process augmented by lipid A. Although greater stimulation of protein synthesis and myristyl acylation could be achieved with longer periods of lipid A exposure, we chose to focus our study on the cohort of proteins appearing during the early phase of the cellular response to lipid A. The patterns of myristyl-acylated proteins in MC and monocytes were, with one notable exception, discordant, suggesting that the acyl proteins are synthesized in a cell-specific fashion.

The process of protein myristyl acylation in these two cell types is dependent upon new protein synthesis, a finding consistent with the cotranslational nature of this process in other cell types (11, 12). The myristate is coupled via amide bonds and, at least in terms of the IL 1 precursors, linked as the intact fatty acid. Although these studies did not specifically address the stoichiometry of the acylation process, the general enhancements in total protein synthesis and in myristyl acylation induced by lipid A were of a similar degree. Differential de-

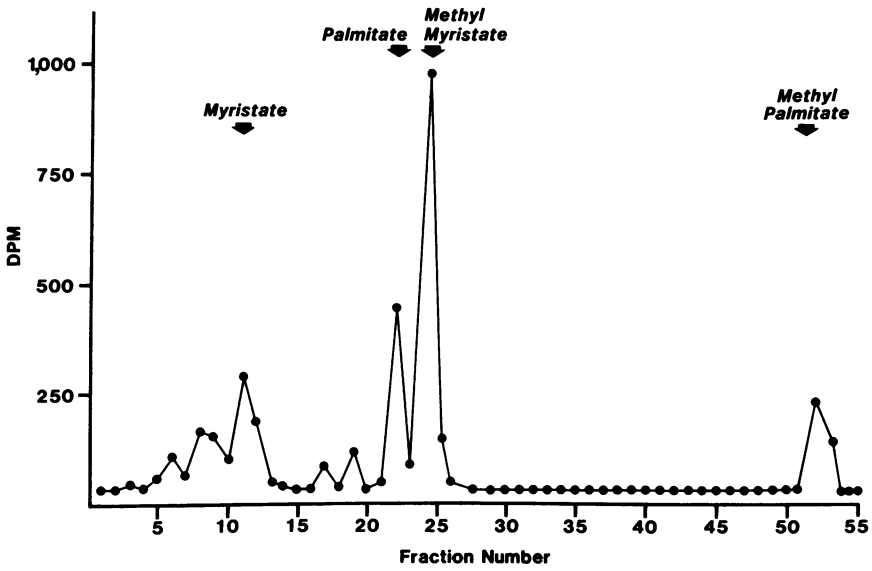

Figure 9. HPLC of the fatty acids covalently bound to the 33-kD monocyte IL 1 precursor proteins. The IL 1 precursor proteins were processed for acid methanolysis of covalently bound fatty acid as detailed in Methods. The elution pattern of radioactivity is compared with the elution pattern of standard fatty acid and fatty acid methyl esters.

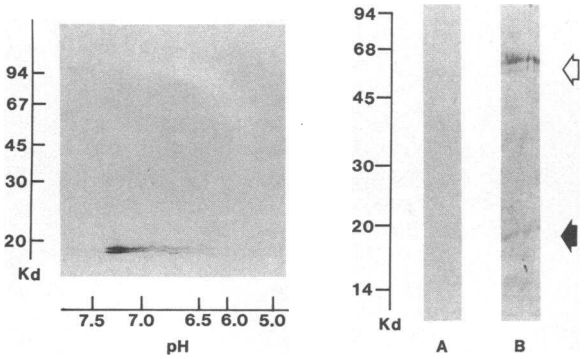

Figure 10. (*A*) Two-dimensional electrophoretic analysis and autoradiography of immunoprecipitated extracellular monocyte IL 1 after 6 h incubation with [^{35}S]methionine and lipid A. (*B*) Immunoprecipitation and Western blot analysis of extracellular monocyte IL 1 after 6 h incubation with [^{3}H]myristate and lipid A. The immune complexes were electrophoresed on 12.5% SDS-PAGE gels and transferred to nitrocellulose. Lane *A*, autoradiogram of the transferred immune precipitate exposed for 3 mo. Lane *B*, Western blot of the same autoradiogram from lane *A*, showing (*solid arrow*) precipitation of the 17-kD extracellular IL 1 protein. *Open arrow*, IgG heavy chain, which is detected by the second antibody (biotinylated goat anti-rabbit IgG) in this Western blot of an electrophoresed immune complex.

grees of acylation in regard to methionine labeling of individual proteins were not observed in either cell type. Our studies suggest that lipid A induces, in a cell-specific fashion, the synthesis of a limited set of cellular proteins. The augmentation of myristyl acylation by lipid A most probably represents enhanced protein substrate availability and not a direct effect of lipid A on the activity of the protein acyl transferase(s) per se. Studies are in progress to compare directly the effects of lipid A on protein versus lysophospholipid acyltransferase activities.

IL 1 is a major secretory product of LPS-stimulated monocytes and mesangial cells (6, 35). Data derived from cloning and sequence analysis for murine IL 1 and human IL 1 α and β have demonstrated that the proteins encoded by the IL 1 mRNA for each species have molecular masses of 30–33 kD (33, 34, 36). To our knowledge, the isoelectric points of the IL 1 α and β precursors have not been determined. In these studies, two 33-kD myristylated proteins with pI of 7.1–7.2 and 7.3–7.4 were identified as the IL 1 α and β precursors, respectively, on the basis of specific immune precipitation. From monocyte supernatants, a single nonacylated protein with the biochemical characteristics of the processed IL 1 β molecule was immunoprecipitated (34, 36). The absence of immunoprecipitable 17-kD IL 1 α in the 6-h stimulated monocyte supernatants may reflect kinetic differences in the translation or processing of this molecule as compared with IL 1 β. The latter is known to be the more abundant IL 1 species produced by stimulated human monocytes (35). Together, the biochemical patterns of IL 1 response detailed here closely parallel the changes in IL 1 biologic activity reported in numerous studies (reviewed in reference 35).

Proliferating human MC express mRNA for both IL 1 α and β in a ratio of $\sim$ 1:5–10 (37). However, the sole translation product detected after peptide growth factor, complement, or LPS stimulation of these cells is the IL 1 β species (6, 37, 38), which is present in human MC lysates as a 33-kD, 7.2-pI precursor protein (37). Thus, the myristylated 33-kD, 7.2-pI rat MC protein observed in these studies presumably represents the acylated mesangial IL 1 β precursor.

Examination of the published amino acid sequences derived from the cDNA for the IL 1 α and β precursors does not provide an immediate indication for the location of the acylated amino acid(s). The fact that the processed, 17-kD form of IL 1 β lacks myristate suggests that the acylation occurs on the NH_2-terminal, 16-kD portion of the 33-kD precursor. Myristyl acylation occurs in the majority of cases on NH_2-terminal glycine residues (21). Penultimate Cys, Asn, Gln, Ala, Val, and Ser[5] facilitate the activity of purified yeast *N*-myristoyl transferase (39, 40). According to published cDNA sequences, neither the IL 1 α or β precursors have an NH_2-terminal glycine after the initiator methionine residue, although actual amino acid sequencing of the 33-kD precursors from intact cells has not been performed. Potential acylation sequences for an *N*-myristoyl transferase include Gly[46] Cys[47] Met[48] Asp[49] Gln[50] Ser[51] in the IL 1 α precursor and Gly[17] Asn[18] Gly[19] Asp[20] Asp[21] within the IL 1 β precursor, which in each case would require proteolytic processing before NH_2-terminal glycine acylation. Alternatively, internal residues such as the ϵ-amino group of lysine may provide sites for myristyl acylation, as suggested for the nicotinic acetylcholine receptor (10, 11) and the μ immunoglobulin heavy chain (22). In the experiments reported here, a small amount of palmitate labeling of the IL 1 precursor molecules was noted. The presence of both myristate and palmitate on the same molecule has been documented in the case of the insulin receptor β chain, even when only one radiolabeled precursor was used (41). This most probably represents two-carbon chain elongation of the myristate precursor to palmitate, with subsequent protein acylation, presumably at a site distinct from the myristyl acylated residue(s) (10).

The mechanisms governing IL 1 synthesis, processing, and secretion have been the object of considerable interest. The IL 1 α and β precursors are unusual both for the absence of a typical hydrophobic leader sequence and the size (16 kD) of the NH_2-terminal portion removed during processing to the mature forms. The NH_2-terminal 16-kD components of the IL 1 α and β precursors have significantly higher degrees of amino acid and structural homology than the rest of the molecules, suggesting that these components perform a conserved biologic function (34). Kurt-Jones et al. (42) have described a membrane-associated form of biologically active IL 1, although the size of this molecule was not determined. Recent studies have localized IL 1 α, which is active in the 33-kD precursor form (43), to the monocyte plasma membrane (25). In contrast, immunoelectron microscopic studies have localized the IL 1 β precursor almost exclusively to the cytoplasmic ground substance of activated monocytes (44). Virtually no IL 1 β precursor was detected in the Golgi apparatus or endoplasmic reticulum, suggesting that IL 1 secretion involves a novel secretory pathway. This intracellular pattern of IL 1 β distribution is compatible with synthesis on free polyribosomes, as suggested for the synthesis of other myristylated proteins (45). Recent evidence indicates that a substantial portion of the cytoplasmic IL 1 β precursor is in fact sedimentable and is associated with microsomes as an integral membrane protein (46). Although smaller than typical secretory vesicles, elliptically shaped clusters containing multiple IL 1 β precursor molecules have been identified within the cytoplasm of activated monocytes (44); such clusters may represent the membrane-associated fraction of IL 1 β noted above.

Based on these considerations, we suggest that the myristyl acylation of the IL 1 precursors on the NH_2-terminal 16-kD component may provide the basis for targeted membrane localization or intermolecular packing of these molecules. Variable acylation of the IL 1 α or β precursors could provide a means for directed localization of these proteins to specific cellular compartments. Membrane-associated or lysosomal proteolytic processing may then result in the release of the 17-kD, nonacylated forms of IL 1 (47, 48). Recent evidence for the selective phosphorylation of the NH_2-terminal component of the IL 1 α precursor molecule suggests that this portion of the molecule has important regulatory significance (49). The specific myristyl acylation of the IL 1 precursor molecules provides a useful and highly relevant protein model for the further analysis of this unusual secretory process and its role in lipid A-mediated cellular activation.

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