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Biochemistry

The 31-kDa precursor of interleukin 1α is myristoylated on specific lysines within the 16-kDa N-terminal propiece

FRAZIER T. STEVENSON*, STUART L. BURSTEN†, CHRISTIE FANTON*, RICHARD M. LOCKSLEY‡, AND DAVID H. LOVETT*§

*The Medical Service, San Francisco Veterans Administration Medical Center, San Francisco, CA 94121; †Department of Medicine, University of Washington/Seattle Veterans Administration Medical Center, Seattle, WA 98108; and ‡Department of Medicine, University of California, San Francisco, CA 94143

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ABSTRACT **The cytokine interleukin 1α (IL-1α) is a critical mediator of the immune and inflammatory responses. A unique determinant of its activity as compared with IL-1β may be its association with the plasma membrane. While the biologic activity of "membrane IL-1" has been extensively reported, the mechanism of membrane binding remains unclear. We report that the N terminus of the 31-kDa IL-1α precursor is myristoylated on specific internal lysine residues. Immunoprecipitation of [^{3}H]myristic acid-radiolabeled human monocyte lysates with IgG antibodies to the 31-kDa IL-1α precursor recovered a protein with the physicochemical properties of the IL-1α N-terminal propiece (16 kDa, pI 4.45). Glycyl N-myristoylation of this protein is precluded by the absence of a glycine residue at position 2, suggesting that the propiece is myristoylated on ε-amino groups of lysine. To determine which lysine(s) are acylated, a series of synthetic peptides containing all lysines found in the IL-1α N-terminal propiece were used in an *in vitro* myristoylation assay containing peptide, myristoyl-CoA, and monocyte lysate as enzyme source. Analysis of the reaction products by reverse-phase HPLC and gas-phase sequencing demonstrated the specific myristoylation of Lys-82 and Lys-83, yielding predominantly monoacylated product. A conserved sequence in the IL-1β propiece was myristoylated with at least 8-fold less efficiency. Acylation of the IL-1α precursor by a previously unrecognized lysyl ε-amino N-myristoyltransferase activity may facilitate its specific membrane targeting.**

N-myristoylation of newly translated proteins has received significant attention as a major determinant of protein targeting and function (for review, see ref. 1). A variety of viral and mammalian membrane-associated proteins are myristoylated; when mutated to nonmyristoylated forms these become soluble, cytosolic proteins with significantly altered function (2–8). For most of these proteins, cotranslational acylation is performed by the enzyme myristoyl CoA:protein *N*-myristoyltransferase, which forms an amide bond between myristic acid and an N-terminal glycine residue. However, a few myristoylated proteins, including the insulin receptor, the μ immunoglobulin heavy chain, tumor necrosis factor α, and the interleukin 1α and 1β (IL-1α and IL-1β) precursors, lack glycine residues correctly positioned for N-myristoylation (9–12). An alternative mechanism for myristoylation of these proteins would be the acylation of internal lysine residues, in which the free ε-amino groups form the characteristic amide bonds. While an enzymatically catalyzed fatty acid (octanoyl) acylation of internal lysine ε-amino groups has been documented for *Agistrodon* phospholipase A_2 (13), discrete cotranslational myristoylation of internal lysine residues has not been demonstrated.

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IL-1α and IL-1β are cytokines with important roles in inflammation and the immune response. Both IL-1α and IL-1β are translated as 31- to 33-kDa precursors which are subsequently proteolytically processed to the extracellularly active, 17-kDa receptor-binding proteins (14–17). Although both forms exhibit virtually identical biologic activities, the 31-kDa IL-1α precursor, in contrast to the IL-1β precursor, also exists as a plasma membrane-associated protein (13–19). Previous studies have demonstrated the myristoylation via amide bond formation of both IL-1α and IL-1β precursors; however, the IL-1α precursor appeared to be the predominant myristoylated species (12). Given the important functional linkage between myristoylation and cellular targeting, we sought to define the sites at which myristoyl amide bond formation occurs within the IL-1 precursor. We report that the acylation of the 31-kDa IL-1 precursors is catalyzed by a specific lysyl myristoyltransferase. Amide bond formation occurs with the ε-amino groups of highly conserved lysines in the N-terminal 16-kDa propiece of each precursor. In addition, these studies demonstrated a preferential myristoylation of the IL-1α precursor, which may facilitate its localization to the plasma membrane.

MATERIALS AND METHODS

Reagents. Lipid A purified from *Salmonella minnesota* R595 was obtained from Ribi Immunochem and prepared as a stock solution (1 μg/ml) in RPMI 1640 supplemented with 0.1% defatted bovine serum albumin. Immediately before use, the stock solution was briefly sonicated on ice. Pansorbin (fixed, protein A-bearing *Staphylococcus aureus*) for radioimmunoprecipitation was obtained from Calbiochem–Behring. Media and heat-inactivated fetal bovine serum were from GIBCO. [^{3}H]Myristate (22 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. An affinity-purified rabbit polyclonal IgG prepared against recombinant 31-kDa IL-1α precursor was the generous gift of R. Black, Immunex.

Preparation of Cells and Cytosol. Human monocytes were harvested and incubated as described (12). For preparation of crude cytosolic fractions used in the *in vitro* myristoylation assay, monocytes were suspended in TE buffer (10 mM Tris·HCl, pH 7.6/1 mM EDTA) with protease inhibitors (5 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 2 μM pepstatin) and subjected to three freeze–thaw cycles at −80°C. Unlysed cells and nuclei were removed by centrifugation at 2000 × *g* and lysates were stored at −80°C.

Radioimmunoprecipitation of Monocyte Lysates. Monocytes were stimulated with lipid A (100 ng/ml), incubated with [^{3}H]myristate (25 μCi/ml), and sonicated (12). Precleared lysates were incubated for 18 hr at 4°C with rabbit polyclonal IgG antibody to recombinant 31-kDa IL-1α. Non-

Abbreviation: IL, interleukin.

§To whom reprint requests should be addressed.

immune rabbit IgG was the negative control. Immunoprecipitates were analyzed by two-dimensional electrophoresis (12, 20, 21). Isoelectric points were determined by comparison with stained protein standards (Pharmacia/LKB).

***In Vitro* Acylation of Synthetic Peptides.** A series of synthetic peptides spanning all lysine residues in the N-terminal 16-kDa propiece of IL-1α were prepared by solid-phase synthesis and purified by reverse-phase HPLC: peptide no. 1, MAKVPDMFED (aa 1–10); 2, PDMFEDLKNCYSENE (aa 5–19); 3, IDHLSLNQKSFYHVSYG (aa 25–41); 4, SETSKTSKLTFKESMV (aa 57–72); 5, TNGKVLKKRRLSLSQ (aa 76–90); 6, SEEEIIKPR (aa 104–112). An IL-1β peptide corresponding to the region covered by IL-1α peptide 5 was also synthesized: VVVAMDKLRKMLVP (aa 67–80). Chemically myristoylated standards were prepared by reaction of each synthetic peptide with the symmetric anhydride of myristic acid (22). The chemically acylated standard peptides were treated with 1 M hydroxylamine to cleave any ester-linked fatty acid, extracted with petroleum ether, and analyzed by reverse-phase HPLC and gas-phase sequencing (see below).

The assay for enzymatic myristoylation of synthetic peptides was based on the method of Towler and Glaser (22). In brief, myristoyl-CoA was prepared by reacting 5 nmol of myristic acid with 10 nmol of LiCoA in an acylation buffer (10 mM Tris·HCl, pH 7.4/0.1 mM EDTA/1 mM dithiothreitol/5 mM $MgCl_2$/5 mM ATP). Thereafter, 15 milliunits of *Pseudomonas* acyl-CoA synthetase (Sigma) were added and the mixture was incubated for 30 min at 30°C in 50 μl. To this was subsequently added 10 nmol of synthetic peptide and 50 μg of monocyte cellular lysate protein in 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA/1 mM dithiothreitol. Protease inhibitors (leupeptin, 8 μM; phenylmethanesulfonyl fluoride, 1 mM; pepstatin, 10 μg/ml) were added and the volume was brought to 110 μl. The enzymatic acylation of the synthetic peptides was continued for up to 10 min at 30°C, followed by the addition of 110 μl of methanol and 10 μl of 100% (wt/vol) trichloroacetic acid. This mixture was incubated on ice for 10 min to precipitate cellular proteins and centrifuged for 10 min at 10,000 × *g*. Supernatants (containing the synthetic peptides) were extracted with petroleum ether three times to remove unreacted myristic acid prior to analysis by reverse-phase HPLC. Standard and chemically acylated synthetic peptides were used to calibrate a C_4 RP304 (Bio-Rad) reverse-phase HPLC column (4.6 × 250 mm) using a linear gradient of acetonitrile (1%/min) in 0.1% trifluoroacetic acid. The column eluates were monitored at 214 nm.

Gas-phase sequencing was performed to confirm the sites of peptide myristoylation after the enzymatic reaction. Derivitized (acylated) lysine residues were indicated by a reduced yield of nonderivatized amino acid detected by the sequencer. In addition to this indirect determination, a fraction of the products from the gas-phase sequencer was diverted during each cycle and directly analyzed for N^{ϵ}-myristoyllysine content by a quantitative HPLC assay. For this assay, standard N^{ϵ}-myristoyllysine was prepared by reaction of N^{α}-(*t*-butoxycarbonyl)lysine (Sigma) with the symmetric anhydride of myristic acid as above, followed by removal of the *t*-butoxycarbonyl protecting group by trifluoroacetic acid hydrolysis. Standard N^{ϵ}-myristoyllysine was used to calibrate a Brownlee ODS-222 column (150 × 2.1 mm) using a linear gradient of acetonitrile (1%/min) in water containing 0.1% trifluoroacetic acid and 0.06% triethylamine. The column eluate was monitored at 214 nm; N^{ϵ}-myristoyllysine was eluted at 51% acetonitrile.

RESULTS

Previous studies (12) demonstrated the incorporation, via amide bond formation, of myristic acid into the intracellular 31-kDa IL-1α and -1β precursors, but not the extracellular, mature 17-kDa C-terminal portions. This suggested that myristoylation occurred within the N-terminal 16-kDa propieces. To precisely locate the site(s) of IL-1α precursor myristoylation, we immunoprecipitated lysates of [^{3}H]myristate-labeled human monocytes by using a polyclonal antibody that recognizes multiple epitopes within the 31-kDa IL-1α precursor. Two myristoylated proteins were recovered: the intact 31-kDa IL-1α precursor protein with pI 4.75 and a protein of ≈16 kDa with a pI of 4.45 (Fig. 1). The physiochemical properties of the smaller molecule correspond to those predicted for the N-terminal propiece of IL-1α, demonstrating that myristoylation of IL-1α takes place within the N-terminal 16-kDa propiece.

Nearly all myristoylated proteins studied to date are acylated on N-terminal glycine residues. Examination of the amino acid sequence of the IL-1 precursors did not reveal a glycine in position 2 which could function as a substrate for N-terminal myristoylation. We therefore postulated that myristoylation and amide bond formation must occur at an available internal amino group, such as the ϵ-amino group of lysine. The human IL-1α precursor N-terminal propiece contains nine lysine residues. To determine which of these residues were myristoylated, we applied an *in vitro* assay (22) for the characterization of the glycine-specific *N*-myristoyltransferase. In this assay, synthetic peptides containing potentially reactive amino acids are combined with myristoyl-CoA and a source of acyltransferase (usually a cell lysate). Cellular proteins are precipitated with trichloroacetic acid/methanol, free myristic acid is extracted with petroleum ether, and the resultant supernatants are analyzed by reverse-phase HPLC. Myristoylated peptides are more hydrophobic and are eluted from reverse-phase columns later than their unmodified forms. Enzymatically myristoylated peptides were identified by comparison with chemically myristoylated standards, which had been made by reacting each peptide with the symmetric anhydride of myristic acid. For the IL-1α analysis, six synthetic peptides of 10–15 amino acids which spanned all the lysine-containing sequences in the N-terminal IL-1α propiece were synthesized (see *Materials and Methods*) and evaluated in the above assay. As a source of a potential ϵ-amino *N*-myristoyltransferase, lysates from lipopolysaccharide-stimulated human monocytes were used. The reaction mixtures were then analyzed for enzymatically myristoylated peptides by reverse-phase HPLC.

Fig. 2 shows typical HPLC elution patterns of several chemically myristoylated peptide standards. The elution profile of unmodified IL-1α peptide 5 is shown in Fig. 2*A*.

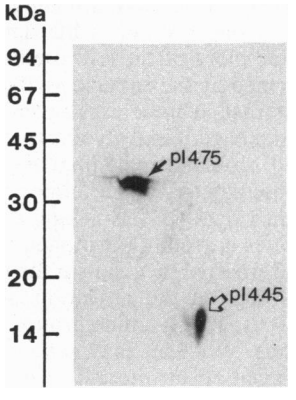

FIG. 1. Autoradiogram immunoprecipitated myristoyl-IL-1α. Human monocytes were labeled with [^{3}H]myristate and the cell lysate was immunoprecipitated with rabbit IgG antibody to recombinant 31-kDa IL-1α. Two prominent proteins are identified: the 31-kDa IL-1α precursor with pI 4.75 (solid arrow) and a 16-kDa protein of pI 4.45 (open arrow), which represents the N-terminal propiece of IL-1α.

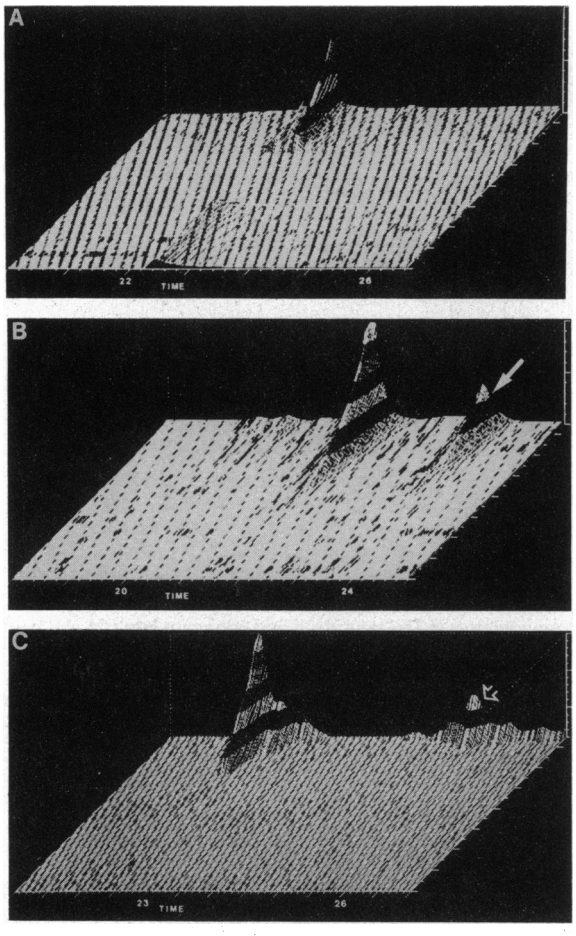

FIG. 2. *In vitro* chemical acylation of IL-1α peptide 5. (*A*) Reverse-phase HPLC elution pattern of IL-1α peptide 5. (*B*) Elution pattern of chemically monoacylated IL-1α peptide 5 (arrow), which is more hydrophobic than the unmodified peptide. (*C*) Elution pattern of chemically diacylated IL-1α peptide 5 (open arrow).

Chemical acylation of this peptide, which contains two contiguous lysine residues, yielded a single, later-eluted peak (Fig. 2*B*), which was shown by gas-phase sequencing and myristoyllysine determination to consist entirely of monoacylated peptide. A second chemically acylated product (Fig. 2*C*) was identified as the diacylated form of this peptide. Fig. 3*A* shows the elution profile of IL-1α peptide 5 after *in vitro* enzymatic acylation for 2 min. The first peak is the unmodified peptide; the second peak demonstrates that the peptide has been rapidly converted to a monoacylated product. In a 5-min reaction, a small amount of diacylated product was formed in addition to the predominant monoacylated derivative (Fig. 3*A Inset*). In contrast to peptide 5, none of the other five IL-1α peptides could be enzymatically acylated (data not shown), indicating that the IL-1α myristoylation site(s) are entirely contained within peptide 5—i.e., on Lys-79, -82, or -83. Unique among the six IL-1α peptides evaluated, peptide 5 exhibited strong amino acid homology to a corresponding sequence in the N-terminal region of IL-1β. Fig. 3*B* demonstrates the *in vitro* enzymatic acylation of this corresponding IL-1β peptide. Monoacylated product was formed, but the yield was considerably less than that obtained with IL-1α peptide 5 (cf. Fig. 3*A* and see below).

A kinetic analysis of the enzymatic monoacylation of IL-1α peptide 5 (Fig. 4) demonstrated the rapid synthesis of the myristoylated product. The reaction rate was significantly blunted by 5 min, at which time nearly 3 nmol of monoacylated product had accumulated.

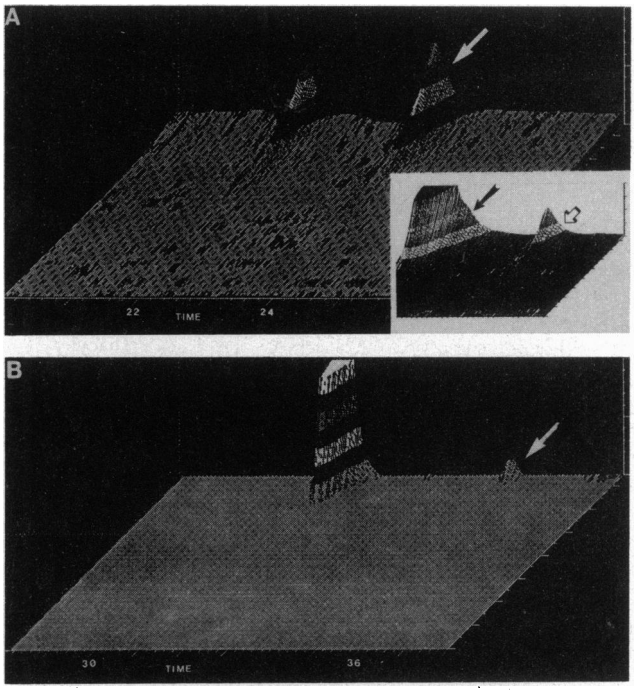

FIG. 3. *In vitro* enzymatic acylation of IL-1 peptides. (*A*) Two-minute enzymatic acylation of IL-1α peptide 5. Note formation of exclusively monoacylated product (arrow). *Inset* shows detail of elution pattern following a 5-min enzymatic acylation. In addition to major monoacylated product (black arrow), diacylated peptide (white arrow) was formed. (*B*) Enzymatic acylation of IL-1β peptide. Reaction product is identified as the monoacylated peptide (arrow).

IL-1α peptide 5 contains three lysine residues, yet in the enzymatic assay, primarily monoacylated product was obtained. To identify which of these three lysine residues was acylated, gas-phase sequencing was performed on pooled preparations of enzymatically mono- or diacylated products. The yields of nonderivatized lysine in cycle 8 (monoacylated product) and in cycles 7 and 8 (diacylated product) were

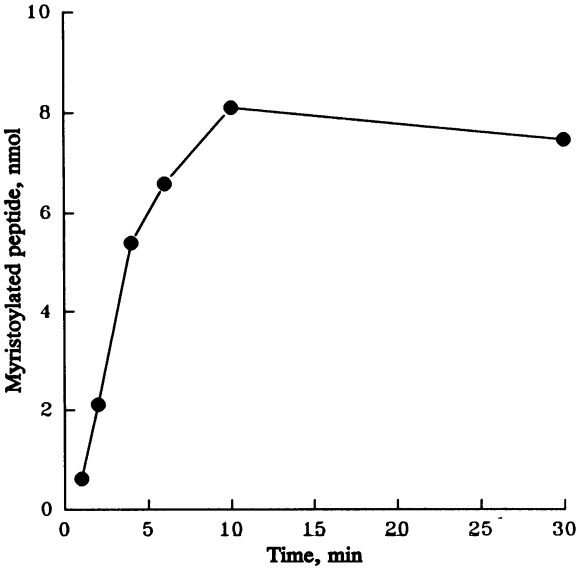

FIG. 4. Kinetics of the enzymatic formation of monoacylated IL-1α peptide 5. The *in vitro* acylation assay was performed as detailed in *Materials and Methods*, except that the reaction was ended at the times indicated. Results are given as the means of triplicate determinations (SD <15% of the mean) and are expressed as nanomoles of monoacylated end product.

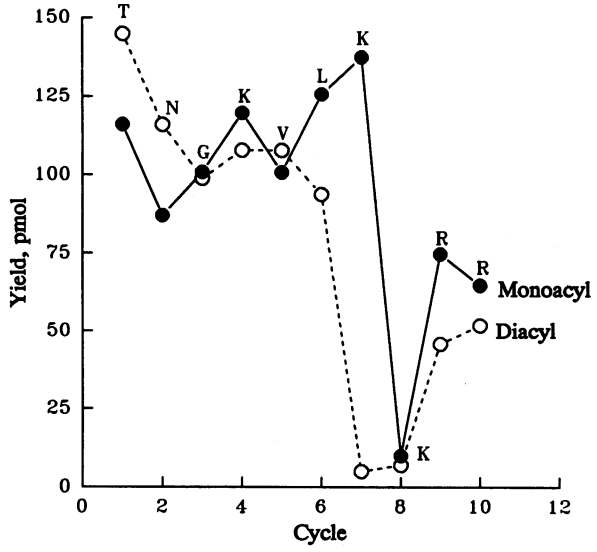

FIG. 5. Recovery of nonderivatized lysine by gas-phase sequencing of enzymatically monoacylated (●) and diacylated (○) IL-1α peptide 5. The yield of nonderivatized (nonacylated) lysines is decreased due to the conversion to acylated, hydrophobic forms. One-letter amino acid symbols are shown.

significantly decreased (Fig. 5), consistent with the conversion of the lysines at these sites to the acylated forms. To confirm the myristoylation of these lysine residues, the N^{ε}-myristoyllysine content of each fraction from gas-phase sequencing was determined in a quantitative HPLC assay (Fig. 6). For the diacylated product, there was little recovery of N^{ε}-myristoyllysine until cycles 7 and 8 (Lys-82 and Lys-83), at which time recovery significantly increased. This corresponds to the observed drop in unmodified lysine signal shown in Fig. 5. These experiments demonstrate two specific points: (*i*) IL-1α peptide acylation is specific for myristoylation of the ε-amino group of lysine and (*ii*) the preferentially myristoylated residue is Lys-83. Quantitation of myristoylated peptide yields (Fig. 7) emphasizes the specificity of this process. Myristoylation favors the production of mono- over diacylated IL-1α peptide 5 by a ratio of ≈20:1 and of

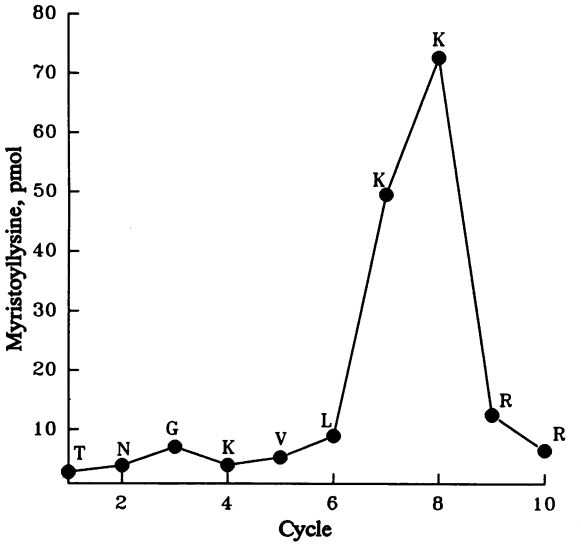

FIG. 6. Identification by reverse-phase HPLC of N^{ε}-myristoyllysine in diacylated IL-1α peptide 5. Myristoyllysine is mainly recovered in gas-phase sequencing cycles 7 and 8, corresponding to residues Lys-82 and Lys-83.

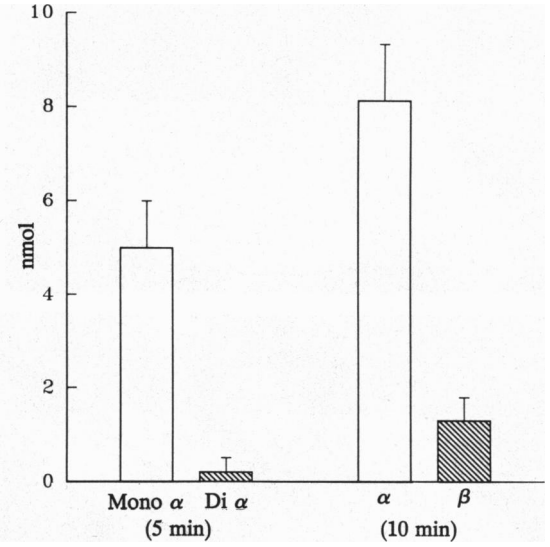

FIG. 7. Comparative yields of enzymatic myristoylation of IL-1 peptide. The recoveries of mono- and diacylated IL-1α peptide 5 and of monoacylated IL-1α peptide 5 and monoacylated IL-1β peptide are shown. Reaction times are in parentheses.

monoacylated IL-1α over IL-1β by nearly 8:1. This indicates that peptide substrate structural determinants control the rates and patterns of myristoylation and that the preferred product is IL-1α monoacylated on Lys-83.

DISCUSSION

In this paper we have provided evidence for the existence of a peptide-lysine ε-amino N-myristoyltransferase activity in monocyte lysates and demonstrated the specific enzymatic myristoylation of two highly conserved lysine residues (Lys-82 and Lys-83) contained within the 112-aa propiece of the 31-kDa IL-1α precursor. Myristoylation of internal lysine residues joins glycyl N-myristoylation, ester-linked palmitoylation, and modification with complex glycosylated phospholipid (23) as documented co- or posttranslational protein acylation mechanisms. Detailed substrate characterization of the glycyl *N*-myristoyltransferase has indicated that it has no activity against lysine (24), suggesting that the lysine-specific acylation observed here represents the action of a distinct enzymatic activity.

The enzymatic octanoylation of two internal lysine residues during the activation of *Agkistrodon* phospholipase A_2 has been reported (13). Acylation led to the conversion of the inactive phospholipase A_2 monomer to a catalytically effective enzyme dimer with enhanced ability to interact with phospholipid monolayers. Similarly, the chemical fatty acylation of lysine ε-amino groups in pancreatic phospholipase A_2 converted the soluble enzyme into a membrane-penetrating form (25). Acylation may play a similar role in the membrane localization of tumor necrosis factor α, a cytokine for which we recently documented myristoylation of specific lysine residues (11).

The IL-1α myristoylation site exhibits strong evolutionary conservation (Fig. 8). The preferentially myristoylated Lys-83 is conserved across all species for which sequence data exist and is contained within a conserved structural motif. We found that the corresponding Lys-76 of IL-1β was myristoylated as well, although the IL-1α peptide was a much better substrate for lysine myristoylation than the IL-1β peptide. This preferential myristoylation supports our previous finding that IL-1α was the predominant myristoylated IL-1 species recovered from lipopolysaccharide-stimulated monocytes (12). Preferential acylation may provide an ex-

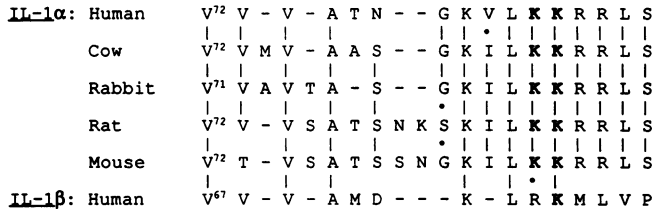

FIG. 8. Interspecies homology of the IL-1α myristoylation site. Myristoylated lysine residues are in bold type. Vertical lines denote strictly conserved residues; dots denote size and charge conservation.

planation for the specific targeting of IL-1α, but not IL-1β, to the cell membrane. Consistent with this, stoichiometric studies comparing the recoveries of [^{35}S]methionine- and [^{3}H]myristate-labeled IL-1α indicate that only 10–15% of the total precursor protein pool is myristoylated (D.H.L., unpublished observations). Secondary-structure analysis predicts a surface exposure of the IL-1α myristoylation site, and epitope analysis using a polyclonal antibody raised against this region has confirmed this supposition (26). An exposed orientation in this region could facilitate processing or enzymatic modifications such as acylation or phosphorylation.

The 31-kDa IL-1α precursor has a complex intracellular distribution, including localization to microtubules, nucleus, cytosol, and the plasma membrane (26). The existence and mechanism of the preferential plasma membrane association of precursor IL-1α have been much debated. IL-1 bioactivity was detected on paraformaldehyde-fixed cells or isolated plasma membranes from a variety of cell types (18, 19, 27–29), but the methodology employed has been criticized (30). Anti-IL-1α monoclonal antibodies stimulated human monocytes to proliferate and to secondarily induce IL-2 expression in T cells, without release of IL-1 bioactivity into the medium (31). Conlon *et al.* (32) demonstrated IL-1α surface localization on peripheral blood monocytes by using monoclonal antibodies and flow cytometry, while our recent work specifically localized the IL-1α precursor to plasma membranes of human mesangial cells (26). Thus, most recent evidence with antibody probes favors the existence of plasma membrane-associated IL-1α in a biologically active form.

Several models for the plasma membrane association of precursor IL-1α have been proposed. Brody and Durum (33) demonstrated that the IL-1α precursor was glycosylated and that it associated with the external surface of the cell membrane by binding to a surface lectin. Kobayashi *et al.* (34, 35) showed that phosphorylation of the IL-1α precursor enabled binding to intrinsic membrane phospholipids. Further, this binding occurred on the inside, but not the outside, surface of erythrocyte ghosts. The IL-1α-precursor myristoylation site (Lys-82 and Lys-83) is located not far upstream from the identified phosphorylation site at Ser-90 (36). Myristoylation in this region could conceivably regulate IL-1α phosphorylation or modulate the ability of the phosphorylated moiety to interact with the cell membrane. Alternatively, myristoylation could be a separate determinant of plasma membrane targeting, acting independently of other protein modifications. This could occur by a simple physicochemical interaction of the myristoyl group with plasma membrane phospholipids or via binding to specific receptors for myristoyl-IL-1α, analogous to those identified for myristoyl-p60^{src} (37). Preliminary studies using dansylated myristoyl acceptor peptides have demonstrated the specific binding of the myristoylated form to the inner leaflet of erythrocyte ghosts, suggesting that acylation facilitates specific membrane binding (D.H.L., unpublished work). Binding of the myristoylated IL-1α precursor to the inner surface of the plasma membrane could facilitate its processing by a Ca^{2+}-dependent neutral protease (19, 27). Membrane targeting of IL-1α may also facilitate its extracellular release either by a Ca^{2+}-dependent mechanism (38) or by passive leakage after cell injury (39). The identification of the specific site of IL-1α myristoylation is a crucial step in determining the role of acylation in the plasma membrane targeting and intracellular trafficking of this important cytokine.

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