Myristyl Acylation of the Tumor Necrosis Factor α Precursor on Specific Lysine Residues

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Summary

NH_2-terminal glycine myristyl acylation is a cotranslational modification that affects both protein localization and function. However, several proteins that lack NH_2-terminal glycine residues, including the interleukin 1 (IL-1) precursors, also contain covalently linked myristate. To date, the site(s) of acylation of these proteins has not been determined. During an evaluation of IL-1 acylation, it was observed that [^{3}H]myristate-labeled human monocyte lysates contained a prominent 26-kD myristylated protein, which was identified as the tumor necrosis factor α (TNF) precursor protein on the basis of specific immune precipitation. Radioimmunoprecipitates from the supernates of labeled monocytes indicated that the processed or mature 17-kD form of TNF does not contain myristate, suggesting that the site of acylation occurs within the 76–amino acid propiece of the precursor molecule. As the TNF precursor does not contain an NH_2-terminal glycine, we hypothesized that myristyl acylation occurs on the N-ϵ-NH_2 groups of lysine, of which two are present in the propiece ($K_{19}K_{20}$). Synthetic peptides were designed to include all seven lysine residues present within the entire 26-kD TNF precursor, and used in an in vitro myristyl acylation assay containing peptide, myristyl-CoA, and monocyte lysate as a source of enzyme. Analysis of reaction products by reverse phase high performance liquid chromatography and gas phase sequencing demonstrated the exclusive myristyl acylation of K_{19} and K_{20}, consistent with the presence in monocytes of a specific lysyl N-ϵ-NH_2-myristyl transferase activity. The acylated lysine residues are located immediately downstream from a hydrophobic, probable membrane-spanning segment of the propiece. Specific myristyl acylation of the TNF propiece may facilitate membrane insertion or anchoring of this critical inflammatory mediator.

Among the many modifications of newly synthesized proteins, cotranslational acylation with myristic acid has received considerable attention as an important determinant of protein function and intracellular localization (for review, see reference 1). For most myristylated proteins studied thus far, acylation occurs via the formation of an amide bond linking the fatty acid to an NH_2-terminal glycine residue after the removal of the initiator methionine. This process has been well characterized and the enzyme responsible, myristyl CoA: protein N-myristyl transferase (NMT),[1] has been cloned (2). However, in a few cases myristylated proteins have been identified that lack the correctly positioned NH_2-terminal glycine strictly required for acylation by NMT. These proteins include the insulin receptor, the μ Ig heavy chain, and the IL-1 α and β precursors (3–5). All of these proteins are myristylated by an undescribed enzymatic mechanism that does not involve acylation on NH_2-terminal glycines. One potential alternative mechanism for myristyl acylation would be the myristylation of internal lysine residues, using the free ϵ-amino groups to form the characteristic amide bonds. Acylation of internal lysine residues with long chain fatty acids has been shown to enhance binding of pancreatic phospholipase A_2 to its substrate (6, 7), but as yet, the myristylation of internal lysine residues as a discrete, cotranslational protein modification has not been demonstrated.

TNF is a cytokine active in mediating cachexia, tumor regression, septic shock, autoimmunity, and complications in infections such as HIV-1, cerebral malaria, and bacterial meningitis (reviewed in reference 8). TNF is translated as a 26-kD precursor molecule that is subsequently processed by unclear mechanisms to an extracellularly active, 17-kD ma-

[1] *Abbreviation used in this paper:* NMT, N-myristyl transferase.

ture protein (9). In addition, the 26-kD TNF precursor protein can act as a plasma membrane–associated protein that mediates inflammation by direct cell-to-cell contact (10, 11). We report here that 26-kD TNF is myristylated and that the acylation occurs via amide bond formation with two specific ε-amino groups of internal lysine residues present within the 76–amino acid propiece of the molecule. Myristyl acylation within this region may facilitate the membrane localization or insertion of the precursor molecule.

Materials and Methods

Reagents. Lipid A, purified from the *Salmonella minnesota* R595 strain, was obtained from Ribi Immunochem (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 FCS were from Gibco Laboratories (Grand Island, NY). [^{35}S]Methionine (1,300 Ci/mmol), [^{35}S]cysteine (800 Ci/mmol), and [^{3}H]myristate (22 Ci/mmol) were obtained from New England Nuclear (Boston, MA). The murine monoclonal anti–human TNF antibody TNF-E was obtained from Genentech Inc. (S. San Francisco, CA). This IgG1 antibody had a neutralization titer of $>5 \times 10^5$ U/ml and an endotoxin concentration of 4 EU/ml by Limulus antilipopolysaccharide assay. Human recombinant TNF was obtained from Genzyme (Cambridge, MA).

Preparation of Cells and Cytosol. Heparinized blood from normal donors was diluted 1:1 with PBS, pH 7.2, before separation over Ficoll-Hypaque cushions (Sigma Chemical Co., St. Louis, MO) by centrifugation for 15 min at 2,000 *g*. The mononuclear cell interface was collected, washed three times in calcium-free PBS, and distributed to 100-mm plastic dishes at a concentration of 5×10^6 cells/ml in RPMI 1640 containing 5% FCS. 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. For preparation of crude cytosolic fractions, monocytes were suspended in TE buffer (10 mM TRIS-Cl, pH 7.6, 1 mM EDTA) containing protease inhibitors (5 mM EDTA, 0.2 mM PMSF, 2 μM pepstatin), and subjected to three cycles of freeze-thawing at −80°C. Unlysed cells and nuclei were removed by centrifugation at 2,000 *g* and lysates were stored at −80°C.

Experimental Protocol. For labeling with [^{35}S]methionine or [^{35}S]cysteine, washed layers of monocytes were incubated for 60 min in methionine- or cysteine-deficient medium. The medium was removed and replaced with fresh medium containing 100 μCi/ml of labeled methionine or cysteine as indicated. Experimental groups were stimulated for 4 h with 100 ng/ml lipid A. After incubation, the culture supernatants were removed, centrifuged at 400 *g* for 10 min, supplemented with protease inhibitors as above, and frozen at −80°C. Monocyte 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. For labeling with [^{3}H]myristate, the fatty acid was dried under argon and brought into solution at 25 μCi/ml by sonication in medium supplemented with 0.1% defatted BSA. After incubation, the cell layers and supernatants were processed as above.

Immune Precipitation. Cell pellets from [^{3}H]myristate-labeled human monocytes were lysed in a 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 above. The lysates were incubated on ice for 30 min, sonicated with 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 cellular TNF, 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 2.5 μg/ml of murine monoclonal anti-TNF IgG. TNF antigen-antibody binding was competed by addition of purified recombinant human TNF in concentrations from 0.1 to 1 μg/ml. Nonimmune murine IgG was used as a negative control. After incubation, 10% Pansorbin was added and the bound immune complexes were recovered by centrifugation. The pellets were washed five times in large volumes of wash buffer containing 0.5% NP-40, 0.45 M NaCl, and 50 mM Tris/HCl, pH 8.3. The pellets were resuspended in 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. The proteins were separated in the first dimension on 0.4 mm ultrathin polyacrylamide gels according to Goldsmith et al. (12), 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% discontinuous gels according to O'Farrell (13). After electrophoresis, the gels were fixed in 10% TCA/30% ethanol for 45 min, washed twice in 5% TCA/30% ethanol, and soaked in Amplify (Amersham Corp., Arlington Heights, IL). Dried gels were analyzed by autoradiography at −80°C using preflashed Kodak X-O-Mat film with intensifying screens. The isoelectric points given were determined by comparison with stained protein standards (Pharmacia LKB, Uppsala, Sweden). Isoelectric points given are not corrected for the inhibitory effects of the 9.2 M urea on hydrogen ion activity and should not be considered identical to isoelectric points obtained under physiologic (i.e., aqueous) conditions (14, 15). Immune precipitation of biosynthetically labeled TNF from the monocyte culture supernatants was performed in an identical fashion and analyzed by one-dimensional electrophoresis on 12.5% SDS-PAGE gels, followed by fixation and autoradiography as above.

Analysis of Acylated Proteins. Recombinant human TNF (10 μg) was added as a carrier protein to [^{3}H]myristate-labeled TNF immune precipitants and separated by SDS-PAGE on 12.5% gels. The TNF protein was identified by Coomassie blue staining, cut out, electroeluted from the gel slice, and concentrated by lyophilization. The samples were subjected to acid methanolysis by heating to 110°C for 60 h in 83% methanol/2 M HCl, containing 200 μg each of myristic and palmitic acid. The reaction products were extracted three times with petroleum ether, and 400 μg each of methyl myristate and methyl palmitate was added. The samples were evaporated under argon, resuspended in methanol, and identified by analytic HPLC on a 4.6 × 25-mm ODS-5 column (Bio-Rad Laboratories, Richmond, CA). The column was developed with 80% (vol/vol) acetonitrile (ACN)/0.1% TFA/0.06% triethylamine (TEA) at a flow rate of 1 ml/min. Serial fractions were collected, and the radioactivity was quantitated by liquid scintillation counting. The elution profile of the radioactivity was compared with the absorbance (210 nm) elution profiles of standard palmitic and myristic acids and the respective methyl esters.

In Vitro Acylation of Synthetic Peptides. A series of synthetic peptides containing potentially reactive lysine residues (Table 1) were prepared and purified by reverse-phase HPLC. Chemically my-

ristylated standards were prepared by reaction of the synthetic peptides with the symmetric anhydride of myristic acid, according to Towler and Glaser (16). 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 enzymatic myristyl acylation of synthetic peptides was based on the method of Towler and Glaser (16). In brief, myristyl CoA was prepared by reacting 5 nmol myristic acid with 10 nmol LiCoA, in an acylation buffer containing 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and 250 nM ATP. Thereafter, 15 mU *Pseudomonas* CoA synthetase (Sigma Chemical Co.) was added and the mixture incubated for 30 min at 30°C in a final reaction volume of 50 μl. To this was subsequently added 10 nmol of synthetic peptide and 50 μg of monocyte cellular lysate in a buffer of 10 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, and 1 mM DTT. A battery of protease inhibitors (8 μM leupeptin, 1 mM PMS-F, and 10 μg/ml pepstatin) was added and the reaction volume was brought to 110 μl. The enzymatic acylation of the synthetic peptides was continued for 10 min at 30°C, followed by the addition of 110 μl methanol and 10 μl saturated TCA. This mixture was incubated on ice for 10 min to precipitate cellular proteins, centrifuged for 10 min at 10,000 *g*, and the supernates (containing the synthetic peptides) were extracted with petroleum ether three times to remove unreacted myristic acid before analysis by reverse-phase HPLC. Standard and chemically acylated synthetic peptides were used to calibrate a 4.6 × 250-mm C4 RP304 (Bio-Rad Laboratories) reverse-phase HPLC column using a linear gradient of ACN (1%/min) in 0.1% TFA. The column eluates were monitored at 215 nm.

To confirm the sites of peptide myristyl acylation after the enzymatic reaction, gas phase sequencing was performed. As myristylated lysine residues are hydrophobic and elute from the sequencer at a higher solvent concentration than nonacylated residues, it is possible to localize within the peptide sequence the actual site of acylation (as determined by the reduced yield of nonderivatized amino acid). 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-NH_2-myristyl lysine content, using a quantitative HPLC assay. For this assay, standard N-ε-NH_2-myristyl lysine was prepared by reaction of N-α-BOC-lysine (Sigma Chemical Co.) with the symmetric anhydride of myristic acid as above, followed by removal of the N-α-BOC protecting group by TFA hydrolysis. Standard N-ε-NH_2-myristyl lysine was used to calibrate a 150 × 2.1-mm ODS-222 column (Brownlee Labs, Santa Clara, CA), using a linear gradient of ACN (1%/min) in a buffer consisting of water/0.1% TFA/0.06% TEA. The column elutate was monitored at 214 nm; N-ε-NH_2-myristyl lysine elutes at 51% ACN.

Table 1. *Synthetic Peptides for TNF In Vitro Myristylation Assay*

Peptide	Sequence
1	E_{14} E A L **K** **K** T G G P Q G S R_{28}
2	R_{83} T P S D **K** P V A H_{92}
3	Y_{136} S Q V L F **K** G Q G_{145}
4	Q_{164} T **K** V N L L S A I **K** S P_{177}
5	G_{185} A E A **K** P W Y E P_{194}

The amino acid sequences of the five synthetic peptides designed to span all lysine residues (*boldface*) in the TNF precursor are shown.

Results

Prior studies from our laboratories have documented the cotranslational myristyl acylation of the intracellular monocyte IL-1 α and β precursor proteins (5). Two-dimensional autoradiograms revealed that multiple intracellular monocyte proteins are myristyl acylated, in addition to the IL-1 precursors. As shown in Fig. 1 *A*, incubation of freshly isolated human monocytes with [^{3}H]myristate for 4 h leads to the labeling of a prominent 25–26-kD protein with an apparent pI of ~6.3. Stimulation of the monocytes for 4 h with 100 ng/ml lipid A before electrophoretic analysis resulted in a three- to fourfold increase in [^{3}H]myristate labeling of the 25–26-kD protein, as determined by quantitative densitometry (Fig. 1 *B*). The myristyl labeling of this particular protein is a cotranslational, or rapid posttranslational event, as no myristyl radiolabeling occurred when the incubations were performed in the presence of 10 μg/ml cycloheximide (Fig. 1 *C*). Concordant labeling of the 25–26-kD protein with [^{3}H]myristate and [^{35}S]methionine was readily apparent using incubation times as short as 1 h (not shown).

Studies by Panuska and colleagues (15), using a similar two-dimensional electrophoresis protocol, have identified a 25–26-kD, pI 6.3 protein present within [^{35}S]methionine-labeled human monocytes as the TNF precursor protein, based on specific immune precipitations. This observation suggested that the prominent 25–26-kD myristate-labeled protein observed in our experiments represented the precursor of TNF. This was confirmed, as shown in Fig. 2 *A*. Using a murine monoclonal anti-human TNF antibody, it was possible to specifically recover the [^{3}H]myristate-labeled 25–26-kD, pI 6.3 protein from monocyte lysates. This recovery was specific and was completely inhibited by competition experiments using an excess of nonlabeled recombinant TNF protein (not shown). We next determined whether the secreted, or processed, 17-kD form of TNF is myristyl acylated. Freshly isolated monocytes were stimulated for 4 h with 100 ng/ml lipid A, during which time radiolabeling was accomplished with either [^{35}S]cysteine or [^{3}H]myristate. The culture supernates were subjected to immune precipitation using the monoclonal anti-TNF antibody and the products analyzed by one-dimensional SDS-PAGE and autoradiography. A [^{35}S]cysteine-labeled, 17-kD protein was readily immune precipitated from the supernates of stimulated monocytes using the monoclonal TNF antibody (Fig. 2 *B*, lane *1*). The molecular mass of this protein is consistent with its identification as the mature, or processed, extracellular form of TNF. In contrast, it was not possible to recover from such supernates a [^{3}H]myristate-labeled 17-kD protein (Fig. 2 *B*, lane *2*). Exposure of such material for periods of 3 mo and greater did not reveal any significant radioactivity in these fractions. These experiments suggest that myristyl acylation is restricted to a component of the 25–26-kD TNF precursor that is removed during the process of secretion.

To confirm that the radiolabel present on the TNF precursor

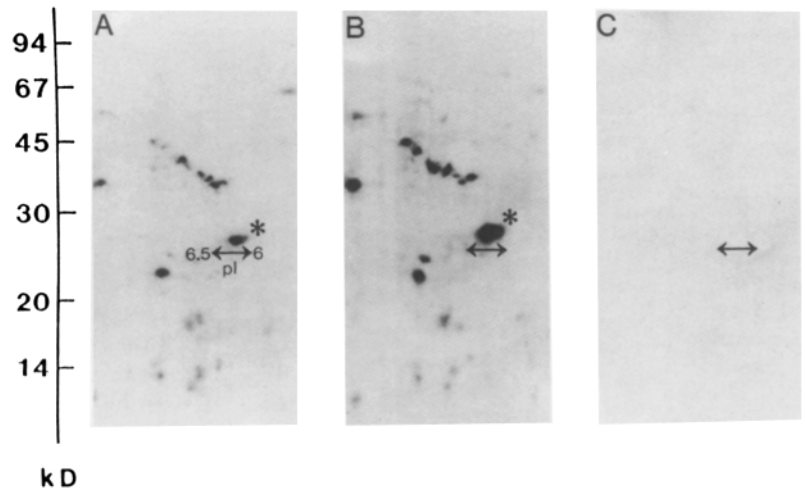

Figure 1. Two-dimensional autoradiograms of human monocyte lysates labeled with [^{3}H]myristate. (*A*) Lysates from freshly isolated monocytes after a 4-h incubation with [^{3}H]myristate. The prominent 26-kD protein (denoted with an asterisk) falls within the pI 6.5–6.0 range. (*B*) Lysates from monocytes incubated for 4 h with 100 ng/ml lipid A in the presence of [^{3}H]myristate. (*C*) Lysates from monocytes incubated for 4 h with 100 ng/ml lipid A, [^{3}H]myristate, and 10 μg/ml cycloheximide.

protein represented intact, covalently linked myristate, acid methanolysis of the isolated [^{3}H]myristate-labeled protein was performed. After recovery of labeled TNF from SDS-PAGE gels, covalently bound fatty acid was hydrolyzed in acid/methanol to form methyl esters suitable for HPLC identification and quantitation. The resultant HPLC separation of the recovered TNF-bound fatty acid is shown in Fig. 3. Approximately 85% of the radioactivity was recovered with the myristyl and methyl myristate fractions. A small amount of radioactivity was identified as either palmitate or methyl palmitate. The myristyl-labeled TNF protein was resistant to hydrolysis with 1 M hydroxylamine (not shown), compatible with linkage in the amide as opposed to thioester form.

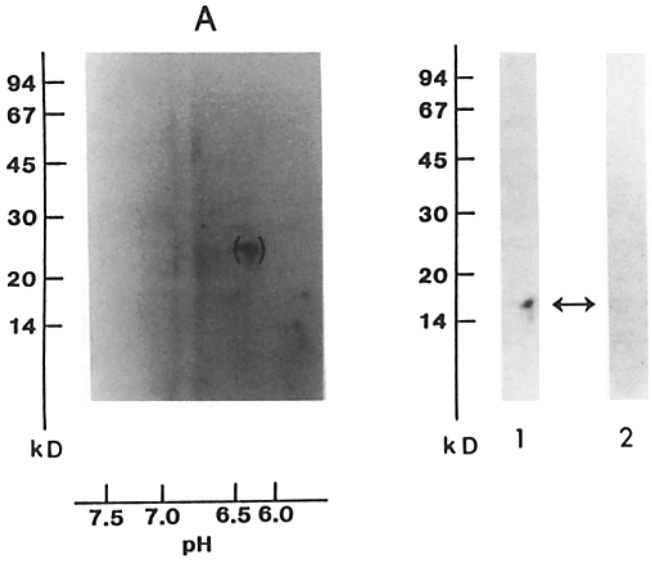

Figure 2. (*A*) Two-dimensional autoradiogram of [^{3}H]myristate-labeled monocyte lysates after immune precipitation with monoclonal anti-TNF IgG. Specifically recovered is the 26-kD, pI 6.5–6.0 TNF precursor protein. (*B*) One-dimensional autoradiogram of [^{35}S]cysteine-labeled monocyte supernates after immune precipitation with monoclonal anti-TNF IgG (lane *1*) with recovery of the 17-kD extracellular TNF protein. A radiolabeled 17-kD TNF protein was not recovered from the supernates of monocytes incubated with [^{3}H]myristate (lane *2*).

These data suggested that myristic acid is covalently linked to the NH_2-terminal propiece of the 26-kD TNF precursor by an amide bond. Nearly all myristylated proteins studied to date have been found to be acylated on NH_2-terminal glycine residues. Examination of the amino acid sequence of TNF did not reveal a glycine in position no. 2 that could function as a substrate for NH_2-terminal myristylation. We therefore considered the possibility that myristylation occurs at an available internal amino group, i.e., the ϵ-NH_2 side group of lysine. The 26-kD TNF precursor contains a total of seven lysine residues. Only two of these ($K_{19}K_{20}$) are contained within the 76–amino acid NH_2-terminal propiece, which we considered the likely site of acylation. To determine which of these seven lysines were myristylated, we used an in vitro assay developed by Towler and Glaser (16) for the characterization of the glycine specific, N-myristyl transferase. In this assay, synthetic peptides containing potentially reactive amino acids were combined with myristyl-CoA and a source of acyltransferase (usually a cell lysate). Cellular proteins were

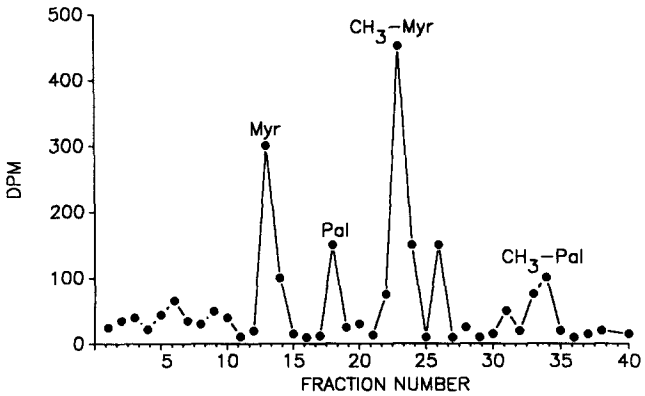

Figure 3. Acid methanolysis of [^{3}H]myristate-labeled TNF. Radiolabeled 26-kD TNF precursor protein was recovered as detailed in Materials and Methods, and subjected to acid methanolysis. The resulting methyl esters and fatty acids were separated by analytic reverse-phase HPLC, and the radioactivity in each fraction was quantified. The bulk (85%) of the radioactivity was recovered as myristate and methyl myristate.

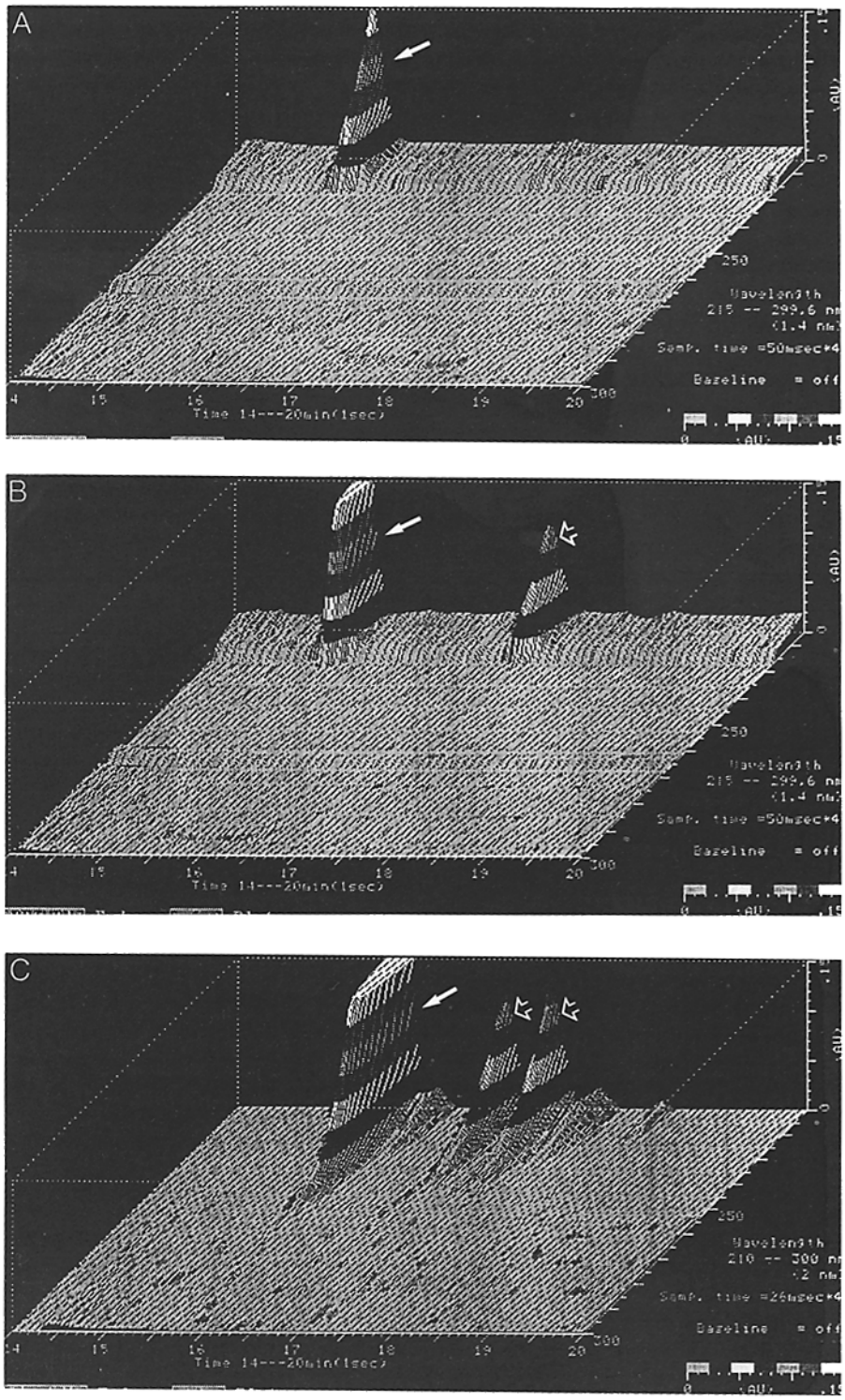

Figure 4. In vitro acylation of TNF peptide no. 1. (*A*) Elution pattern of TNF peptide no. 1 (*filled arrow*) by reverse-phase HPLC. (*B*) Elution pattern of chemically bis-acylated TNF peptide no. 1 (*open arrow*), which is more hydrophobic than the unmodified peptide (*filled arrow*). (*C*) Elution pattern of enzymatically acylated TNF peptide no. 1, revealing the formation of mono- and bis-acylated TNF peptides (*open arrows*).

precipitated with TCA/MeOH, free myristic acid was extracted with petroleum ether, and the resultant supernatants were analyzed by reverse-phase HPLC. Myristylated peptides are more hydrophobic and elute later than their unmodified forms. Enzymatically myristylated peptides were identified by comparison with chemically myristylated standards, which had been made by reacting each peptide with the symmetric anhydride of myristic acid. For the TNF analysis, five synthetic peptides of 10–15–amino acid length, which spanned all the lysine-containing sequences in the 26-kD TNF precursor, were synthesized (see Table 1) and evaluated in the above assay. As a source of a potential N-ε-NH_2-myristyl transferase, cell lysates from LPS-stimulated human monocytes were used. The reaction mixtures were then analyzed

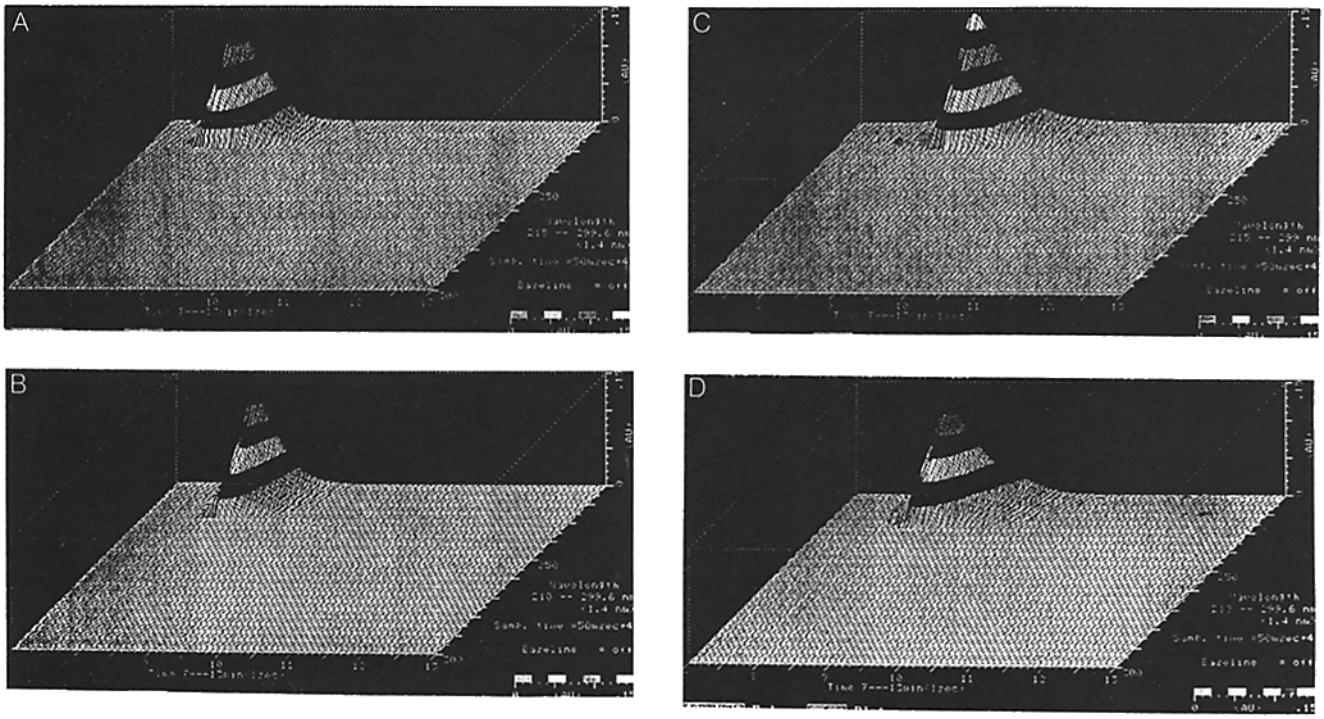

Figure 5. Results of the enzymatic in vitro acylation of TNF peptides 2–5 (*A–D*, respectively). In no case was an acylated end-product detected.

for the presence of enzymatically myristylated peptides by reverse-phase HPLC. In Fig. 4 *A*, the elution profile of TNF peptide no. 1 is shown. Chemical acylation of this peptide, which contains two contiguous lysine residues, yielded a later eluting peak (Fig. 4 *B*), which was shown by gas-phase sequencing and myristyl-lysine determination to consist entirely of bis-acylated peptide. Fig. 4 *C* shows the HPLC analysis of TNF peptide no. 1 after reaction in the enzymatic myristylation assay. The elution profile shows the unreacted peptide peak, as well as a doublet that coelutes with the myristyl peptide standard. This doublet represents the mono- and bis-acylated forms of the peptide (see below). In contrast to the results obtained with TNF peptide no. 1, it was not possible to demonstrate any enzymatic acylation of TNF peptides nos. 2–5 (Fig. 5). To unambiguously confirm the myristyl acylation of the TNF peptide no. 1, gas phase sequencing and direct quantitation of N-ε-myristyl lysine was performed on the later eluting, bis-acylated product. As shown in Fig. 6, the yields of nonderivatized lysine in cycles 6 and 7 were significantly decreased, consistent with the conversion of the lysines to the acylated forms. Direct quantitation of N-ε-NH_2-myristyl lysine confirmed the presence of the derivatized forms in sequencing cycles 6 and 7 (Fig. 7). Analysis of the mono-acylated product of the enzymatic reaction indicated the preferential acylation of the second lysine residue (K_{20}; not shown). A time kinetic analysis of the enzymatic acylation of TNF peptide no. 1 is shown in Fig. 8, which demonstrates the rapid synthesis of the bis-acylated end-product. The reaction rate is significantly blunted by 5 min, at which time nearly 3 nmol of end-product have accumulated.

Discussion

In this paper the specific enzymatic myristylation of two lysine residues contained within the 76–amino acid propiece of the 26-kD TNF precursor protein has been demonstrated. In addition, the studies utilizing synthetic peptides provide evidence for the existence of a lysyl peptide N-ε-NH_2-

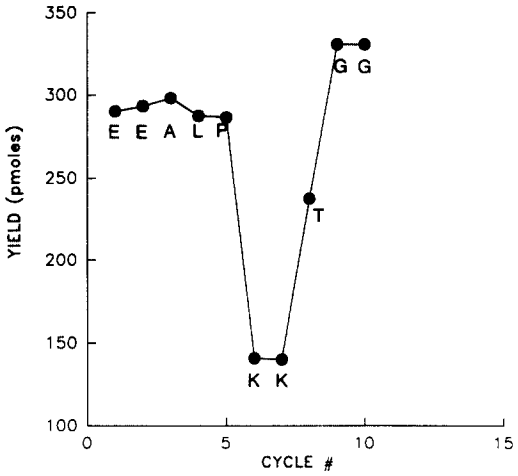

Figure 6. Recovery of nonderivatized lysine by gas-phase sequencing of enzymatically acylated (bis) TNF peptide no. 1. The yield of nonderivatized (i.e., nonacylated) lysines is decreased due to the conversion to acylated, hydrophobic forms. Although the sequencing was performed on HPLC-purified bis-acylated TNF peptide no. 1, there is a partial conversion of the acylated lysine residues during the sequencing reaction (hydrolysis) to the nonacylated forms.

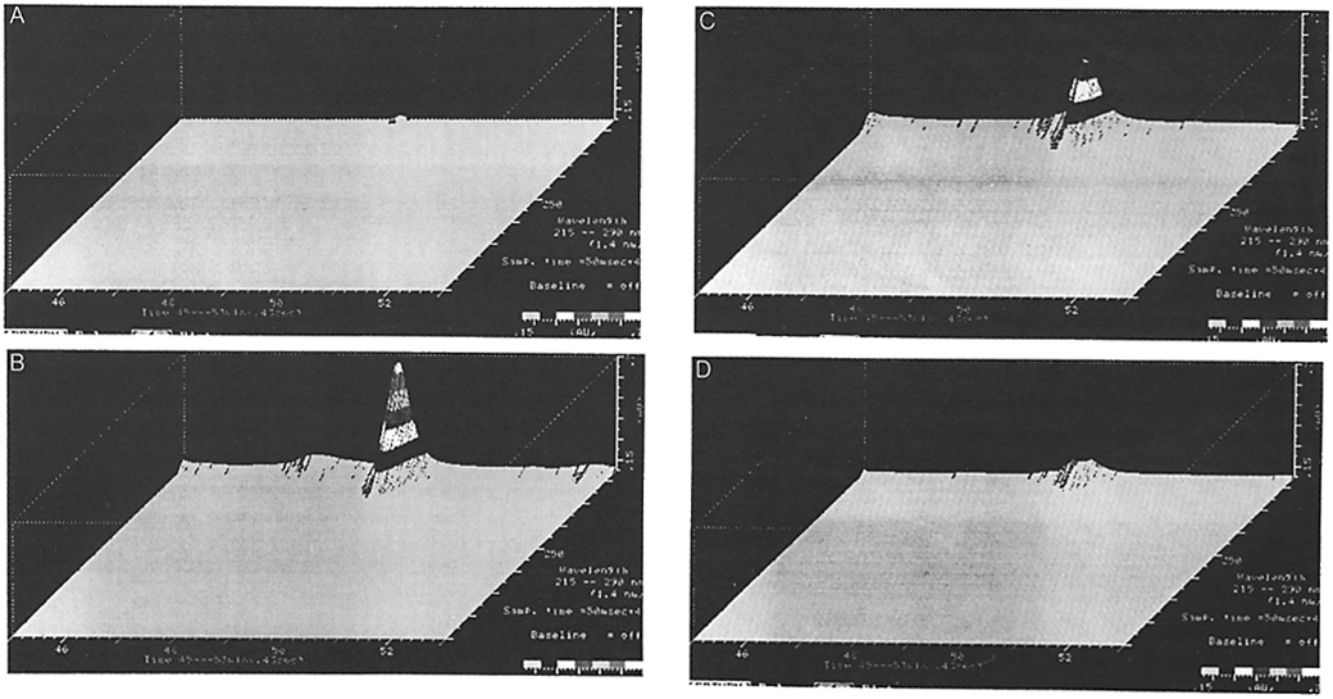

Figure 7. Identification by reverse-phase HPLC of N-ϵ-NH_2-myristyl lysine in bis-acylated TNF peptide no. 1 gas-phase sequencing cycles 6 and 7 (*A–D*, cycles *5–8*, respectively).

myristyl transferase activity. Myristylation of internal lysine residues joins a short list of co- or posttranslational protein acylations that includes N-glycyl myristylation, ester-linked palmitylation, and modification with complex glycosylated phospholipid (17). Further knowledge concerning the structure, substrate specificities, and relationship of the lysyl N-ϵ-NH_2-myristyl transferase to the N-glycyl myristyl transferase awaits its purification and characterization. The detailed substrate characterization of the N-glycyl myristyl transferase has indicated a complete lack of activity against lysine (18), and it must therefore be assumed that the lysyl-specific activity observed here represents a distinct, and previously unrecognized, enzymatic entity. The careful quantitative study of Towler and Glaser (19), concerning the acylation of cellular proteins, indicated that ~70–80% of total amide-linked myristate was in the form of myristyl glycine. Interestingly, a significant amount of radioactivity was also present in an undefined fraction with the HPLC elution properties characteristic of myristyl lysine, suggesting that acylation of this residue may not be a rare event.

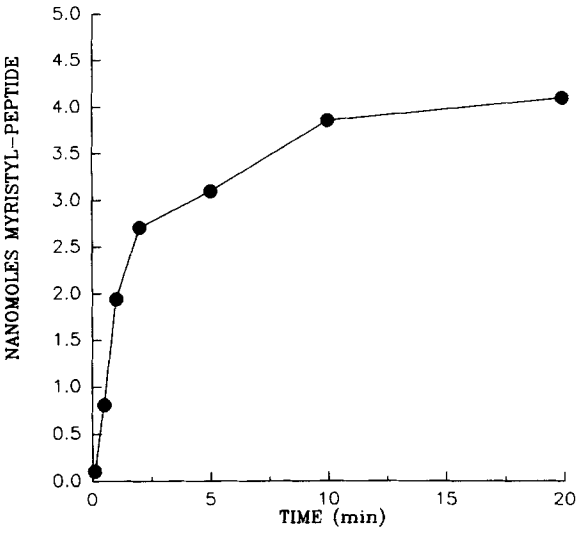

Figure 8. Time kinetics of the enzymatic formation of bis-acylated TNF peptide no. 1. The in vitro acylation assay was performed as detailed in Materials and Methods, with the exception that the reaction was ended at the time points indicated. Results are given as the means of triplicate determinations and are expressed as nanomoles of bis-acylated end-product.

The enzymatic (octanoyl) acylation of two internal lysine residues as a consequence of the activation of *Agkistrodon* phospholipase A_2 has been described (6). The functional consequence of this event was the conversion of the inactive phospholipase A_2 monomer to a catalytically effective enzyme dimer that exhibited enhanced interaction with phospholipid monolayers. Similarly, chemical acylation with a series of fatty acids of lysyl ϵ-NH_2 groups in pancreatic phospholipase A_2 converted the soluble enzyme into a membrane-penetrating form (20). These studies also demonstrated that attachment of acyl groups to hydrophobic regions, as opposed to hydrophilic regions, significantly enhanced the degree of membrane penetration, and that this penetration was optimized by utilizing fatty acids with smaller molecular areas. Further insights into the potential role of the myristyl acylation of lysine residues may be deduced from work done on N-glycyl-

Table 2. *Interspecies Homology of the TNF Myristylation Site*

Species													
Human	E_{14}	E	A	L	P	K	**K**	T	G	G	P	Q	G
	|	|	|	|	|	|	|	·	|	|	|	|	|
Pig	E_{14}	E	A	L	A	K	**K**	A	G	G	P	Q	G
	|	|		|	·		|	|	|	|	|	|	|
Sheep	E_{14}	E	V	L	S	N	**K**	A	G	G	P	Q	G
	|	|		|	·		|	|	|	|	|	|	|
Cat	E_{14}	E	A	L	P	K	**K**	A	G	G	P	Q	G
	|		·	|	|	|	|	|	|	|	|	|	|
Rabbit	E_{14}	G	P	L	P	K	**K**	A	G	G	P	Q	G
	|		·	|	|	|	|		|	|		|	
Rat	E_{14}	E	A	L	P	K	**K**	M	G	G	L	Q	N
	|	|	|	|	|		|		|	|		|	
Mouse	E_{14}	E	A	L	P	Q	**K**	M	G	G	F	Q	N

Diagram demonstrating the interspecies amino acid homology for the region surrounding the myristylated lysine residues (*boldface*). Vertical lines denote strictly conserved residues, dots denote functional conservation.

myristylated proteins. One extensively studied function has been the plasma membrane targeting of myristylated proteins. The viral proteins $p60^{v\text{-}src}$, $Pr65^{gag}$, and a variant of p21 Ras are all myristylated, membrane-associated proteins (21–24). Point mutations that abolish myristylation convert them into soluble, cytosolic proteins, with consequent impairment of transforming ability or viral assembly. A similar role for myristylation has been demonstrated for several mammalian proteins. The protein kinase C substrate proteins and the α subunit of the GTP inhibitory binding protein all require binding of myristic acid in order to associate with the cell membrane (25–27).

TNF-α, active extracellularly as a 17-kD protein, is translated as a 26-kD precursor molecule. Initially, it was thought that the NH_2-terminal propiece represented an unusually long signal peptide, and that the molecule was processed to its mature form through the classical secretory pathway (28). However, Muller et al. (29) found that microsomes failed to process the TNF precursor when analyzed in an in vitro translation system. In 1988, Kriegler et al. (9) demonstrated that the 26-kD TNF precursor protein exists as an integral, transmembrane protein. The orientation of the TNF precursor (N_{cyto}/C_{exo}) was determined by differential proteolytic digestions and is consistent with classification as a type II integral membrane protein (30). Type II proteins contain signal/anchor domains, as opposed to cleavable signal peptides, and this presumably explains the failure of microsomal preparations to process the 26-kD precursor to the 17-kD form (9, 29).

The existence and biologic activity of plasma membrane–associated TNF has been confirmed by subsequent studies. Chensue et al. (31) showed the presence of membrane-associated TNF on mouse peritoneal macrophages by immunohistochemical and electron microscopic techniques. Perez et al. (11) found that transfected cells expressing a noncleavable mutant of 26-kD TNF on the cell surface were active in cell-to-cell killing, which did not require processing to the 17-kD form. In contrast to the findings of Kriegler et al. (9), Bakouche et al. (32) failed to detect an integral membrane 26-kD TNF protein in activated human monocytes, but instead recovered a membrane-associated, salt-elutable 17-kD protein, suggesting that the processed TNF molecule was linked to a discrete, membrane-associated TNF-binding protein. Leuttig et al. (33) found evidence for both mechanisms in murine macrophages. Plasma membranes contained both an acid-elutable 17-kD form of TNF with the characteristics of a receptor-bound protein, and a 26-kD integral transmembrane form. The transmembrane form possessed about 60% of total TNF bioactivity. In summary, there appear to be two forms of biologically active membrane-associated TNF: an integral membrane protein of 26 kD and a processed, 17-kD form presumably bound to a receptor or binding protein. Processing of the 26-kD transmembrane form to the 17-kD form apparently involves the action of proteolytic enzymes located on the cellular surface, (9), leaving behind the propiece within the membrane (34). Structural analysis of the site of myristyl acylation of the TNF propiece places the target lysine residues almost immediately adjacent to a hydrophobic stretch of sufficient length (24 residues) to act as a membrane-spanning or anchoring sequence. Examination of the interspecies homology of the TNF myristylation site shows significant amino acid conservation (Table 2). In particular, the preferentially myristylated $lysine_{20}$ is conserved across all species, consistent with its having a conserved functional role. This site also conforms to the "positive-inside rule" of von Heijne (30), as calculation of the charge distribution across the putative membrane-spanning region is consistent with the experimental delineation by Kriegler (9) of a type II N_{cyto}/C_{exo} orientation. Given these considerations, we hypothesize that the func-

tional significance of the myristylation of these lysines is to facilitate the membrane insertion or anchoring of this sequence. This event could occur primarily as a consequence of a physicochemical interaction of the acyl group with membrane phospholipids or via binding to receptors specific for myristylated TNF, analogous to that identified for myristyl-p60^{src} by Resh and Ling (35).

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