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

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Summary

NH₂-terminal glycine myristyl acylation is a cotranslational modification that affects both protein localization and function. However, several proteins that lack NH₂-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 [³H]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 NH₂-terminal glycine, we hypothesized that myristyl acylation occurs on the N-ε-NH₂ groups of lysine, of which two are present in the propiece (K₁₉K₂₀). 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 K₁₉ and K₂₀, consistent with the presence in monocytes of a specific lysyl N-ε-NH₂-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 NH₂-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 NH₂-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 NH₂-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 A₂ 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-
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Materials and Methods

Reagents. Lipid A, purified from the Salmonella minnesota R595 strain, was obtained from Ribi Immunocomb (Hamilton, MT). 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. Pastebin (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). 35S]Methionine (1,300 Ci/mmol), and [3H]myristate (22 Ci/mmol) were obtained from New England Nuclear (Boston, MA). The murine monoclonal anti-human TNF antibody TNF-E (Cambridge, 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 x 10^3 U/ml and an endotoxin concentration of 4 EU/ml by Limulus antilipopoly- saccharide 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 x 10^6 cells/ml in RPMI 1640 containing 5% FCS. After incubation at 37°C in 5% CO2 for 2 h, nonadherent cells were removed by vigorous washing. The adherent monolayers consisted of >95% monocytes as assessed by nonspecific esterase staining and phagosome assay. Human recombinant TNF was obtained from Genzyme (Cambridge, MA).

Analysis of Acylated Proteins. Recombinant human TNF (10 µg) was added as a carrier protein to [3H]myristate-labeled TNF immuno 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 methanolation 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 x 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-
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 5 nmol LiCoA, in an acylation buffer containing 10 mM Tris/HC1, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 5 mM MgCl2 and 250 nM ATP. Thereafter, 15 mM Pseudomonas Coa synthetase (Sigma Chemical Co.) and the mixture were stirred 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/HC1, 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 (8 µM leupeptin, 1 mM PMS-F, and 10 µg/ml pepstatin) were 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 150 x 2.1-mm ODS-222 column (Brownlee Labs, Santa Clara, CA), using a linear gradient of ACN (0-45%) 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 sequencing was diverted during each cycle and directly analyzed for N-ε-NH2-myristyl lysine content, using a quantitative HPLC assay. For this assay, standard N-ε-NH2-myristyl lysine was prepared by reaction of 2-BOC-lysine (Sigma Chemical Co.) with the symmetric anhydride of myristic acid as above, followed by removal of the N-2-BOC protecting group by TFA hydrolysis. Standard N-ε-NH2-myristyl lysine was used to calibrate a 150 x 2.1 mm 0DS-222 column (Brownlee Labs, Santa Clara, CA), using a linear gradient of ACN (1%/min) in 0.1% TFA. The column elutes were monitored at 215 nm.

Table 1. Synthetic Peptides for TNF In Vitro Myristylation Assay

| Peptide | Sequence |
|---------|----------|
| 1       | E11 E A L K K T G G P Q G S R38 |
| 2       | R35 T P S D K P V A H92 |
| 3       | Y136 S Q V L F K G Q G160 |
| 4       | Q164 T K V N L L S A I K S P177 |
| 5       | G185 A E A K P W Y E P194 |

The amino acid sequences of the five synthetic peptides designed to span all lysine residues (boldface) in the TNF precursor are shown.
Figure 1. Two-dimensional autoradiograms of human monocyte lysates labeled with [3H]myristate. (A) Lysates from freshly isolated monocytes after a 4-h incubation with [3H]myristate. The prominent 26-kD protein (denoted with an asterisk) falls within the pl 6.5-6.0 range. (B) Lysates from monocytes incubated for 4 h with 100 ng/ml lipid A in the presence of [3H]myristate. (C) Lysates from monocytes incubated for 4 h with 100 ng/ml lipid A, [3H]myristate, and 10 µg/ml cycloheximide.

These data suggested that myristic acid is covalently linked to the NH2-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 NH2-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 NH2-terminal myristylation. We therefore considered the possibility that myristylation occurs at an available internal amino group, i.e., the e-NH2 side group of lysine. The 26-kD TNF precursor contains a total of seven lysine residues. Only two of these (K19K20) are contained within the 76–amino acid NH2-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...
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-e-NH2-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.

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-e-NH2-
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-e-NH₂-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.

The enzymatic (octanoyl) acylation of two internal lysine residues as a consequence of the activation of Agkistrodon phospholipase A₂ has been described (6). The functional consequence of this event was the conversion of the inactive phospholipase A₂ 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 e-NH₂ groups in pancreatic phospholipase A₂ 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-
brane-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), Bakhouche 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. Leutttig 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 myristoylation 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, 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\textsubscript{myr}/C\textsubscript{exo} orientation. Given these considerations, we hypothesize that the func-

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

| Species | E14 | E | A | L | P | K | K | T | G | G | P | Q | G |
|---------|-----|---|---|---|---|---|---|---|---|---|---|---|---|
| Human  | E14 | E | A | L | P | K | K | T | G | G | P | Q | G |
| Pig    | E14 | E | A | L | A | K | K | A | G | G | P | Q | G |
| Sheep  | E14 | E | V | L | S | N | K | A | G | G | P | Q | G |
| Cat    | E14 | E | A | L | P | K | K | A | G | G | P | Q | G |
| Rabbit | E14 | G | P | L | P | K | K | A | G | G | P | Q | G |
| Rat    | E14 | E | A | L | P | K | M | G | G | L | Q | N |
| Mouse  | E14 | 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.
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-p60src by Resh and Ling (35).

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