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Covalently bound myristate in a lymphoma tyrosine protein kinase

(fatty acylation of proteins/murine lymphoma cells/pp60^{src})

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ABSTRACT The murine lymphoma cell line LSTRA expresses high levels of a membrane-associated tyrosine protein kinase, which we now show to be acylated by [^{3}H]myristate *in vivo*. This [^{3}H]myristate-labeled tyrosine protein kinase is immunoprecipitated from detergent extracts of postnuclear particulate fractions with an antibody directed against its single site of tyrosine phosphorylation. This site has an amino acid sequence also found in the transforming proteins of the Rous sarcoma and Y73 viruses. Preincubation of the antibody with a peptide containing the same sequence completely blocks this immunoprecipitation. The [^{3}H]myristate linkage to the protein is stable in boiling 2% NaDodSO$_4$/0.125 M Tris Cl, pH 6.7/5% 2-mercaptoethanol, which suggests an amide rather than an ester linkage. Analogous attempts to label with [^{3}H]palmitate show negligible incorporation into either nonnuclear particulate proteins or immunoprecipitated proteins. Chemical characterization of the immunoprecipitated protein isolated by NaDodSO$_4$/PAGE verifies that the ^{3}H label is in protein-associated myristate. Sonicated 5% NaDodSO$_4$ extracts of LSTRA and YAC-1 (another murine lymphoma line) cells contain quite different distributions of myristoylated proteins.

Protein kinases that phosphorylate tyrosine residues may be involved in the regulation of cellular proliferation. Receptors for four different hormones or growth factors appear to have tyrosine protein kinase activity (1–5), and certain other mitogenic agents also stimulate the phosphorylation of tyrosine residues in proteins (6–8). Cells transformed by certain RNA tumor viruses possess elevated tyrosine protein kinase activity. A related group of cellular oncogenes encode tyrosine protein kinases (see reviews in refs. 9 and 10).

The Moloney leukemia virus-transformed T-cell line LSTRA has been shown to have elevated tyrosine protein kinase activity, as compared to normal T lymphocytes and to another murine lymphoma line, YAC-1 (11). The high level of tyrosine phosphorylation in LSTRA cells is presumably due to a prominent tyrosine protein kinase of M_r 56,000, which is itself subject to phosphorylation at tyrosine to give the phosphoprotein pp56. This kinase crossreacts with and is inhibited by antibodies raised against a synthetic peptide containing the amino acid sequence encompassing its single tyrosine phosphorylation site (12). This site is also found in the transforming proteins of the Rous sarcoma and Y73 viruses (13). Using this antibody, we now have found that cultured LSTRA cells incorporate [^{3}H]myristate into the tyrosine protein kinase. Myristoylation is a covalent modification previously observed in only a few proteins, several of which are implicated in the phosphorylation or dephosphorylation of other proteins.

The catalytic subunit of bovine cAMP-dependent protein kinase was the first protein shown to contain myristate in amide linkage to amino-terminal glycine (14). The protein phosphatase calcineurin was found to be similarly modified (15). It is known that myristoyl groups are conjugated to the p15 protein derived from Pr65^{gag} encoded by the Moloney murine leukemia virus (16), to various *gag-onc* fusion proteins (17), and to pp60^{src}, the tyrosine protein kinase encoded by the Rous sarcoma transforming gene (B. Sefton, personal communication). The significance of the attachment of long-chain fatty acids to proteins is still a matter of conjecture (14, 18–21), but possible roles as membrane anchors or recognition groups have been suggested. It is known that myristoyl groups are conjugated to amino-terminal glycyl residues in several proteins (14–16), whereas palmitoyl groups are, in some cases, attached by ester bonds (21). Secondary modification of proteins with these two fatty acids may well serve different cellular roles, although little is known about such functions. Thus, it is important to establish whether all tyrosine protein kinases involved in regulation of cell growth are myristoylated and whether such modification is necessary for regulation.

MATERIALS AND METHODS

The preparation of a peptide (Lys-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly) corresponding to the tyrosine phosphorylation site of LSTRA tyrosine protein kinase and the preparation of affinity-purified antibodies to that peptide are described elsewhere (12).

[9,10-^{3}H]Myristic acid was prepared by Amersham by reduction of myristoleic acid (*cis*-9-tetradecenoic acid, Sigma) with 5 Ci (185 GBq) of tritium. The crude product mixture was dissolved in toluene and stored at −20°C in 5-mCi aliquots containing ≈0.25 μmol of myristic acid, which were evaporated to dryness and redissolved in 200 μl of acetonitrile (Burdick and Jackson, Muskegon, MI) containing 0.08% trifluoroacetic acid (Pierce). The [^{3}H]myristic acid was purified by applying this solution to a TSK C_{18} (Toyo Soda) reversed-phase HPLC column, equilibrated in 60:40:0.09 acetonitrile/water/trifluoroacetic acid. Elution was carried out at a flow rate of 2 ml/min; the concentration of acetonitrile (containing 0.08% trifluoroacetic acid) was increased linearly from 60 to 100% over 30 min. [^{3}H]Myristic acid was eluted at ≈80% acetonitrile. The acetonitrile/water azeotrope was largely removed by gentle heating at 77°C in a fume hood.

The LSTRA and YAC-1 murine lymphoma cell lines were maintained in culture as described (11, 22). For labeling studies, ≈4 × 10^7 lymphoma cells were collected at 300 × g_{max} and then washed in RPMI 1640 (GIBCO), pH 7.4, containing L-glutamine (GIBCO, 0.3 g/liter), sodium pyruvate (GIBCO,

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Abbreviations: pp56, phosphorylated form of the prominent tyrosine protein kinase of cells of the murine lymphoma line, LSTRA; pp60^{src}, phosphorylated form of the Rous sarcoma virus transforming-gene product.

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0.11 g/liter), $NaHCO_3$ (3 g/liter), and 2-mercaptoethanol (3.4 μl/liter). The cells were dispersed in 20 ml of the above medium containing 25 μCi of [^{3}H]myristate or [9,10-^{3}H]-palmitate (17 Ci/mmol, New England Nuclear) per ml and placed in a 150-mm maxiplate (Falcon) in a humidified 37°C incubator in an atmosphere of 5% carbon dioxide for 4 hr.

For immunoprecipitations, LSTRA cells from one maxiplate were collected by centrifugation as above, washed once with 10 ml of ice-cold phosphate-buffered saline (pH 7.4), recentrifuged, and then homogenized by 20 strokes of the A pestle in a Wheaton Dounce homogenizer containing 1 mM $MgCl_2$/5 mM 2-mercaptoethanol/5 mM Hepes, pH 7.4. Nuclei were removed by centrifugation at 1300 × g_{max} for 1 min. A postnuclear particulate fraction was then obtained by centrifugation at 300,000 × g_{max} for 45 min. This pellet was resuspended in 0.1 ml of homogenization buffer and then extracted by the addition of 0.1 ml of 2% Triton X-100/2% sodium deoxycholate/0.2% $NaDodSO_4$/0.3 M NaCl/0.2 M Tris Cl, pH 7.2. After centrifugation, aliquots of this particulate fraction extract were incubated for 3 hr at 0°C with various quantities of antibody directed against the single site of tyrosine phosphorylation in LSTRA tyrosine protein kinase (12). Immunocomplexes were collected by centrifugation after the addition of 75 μl of a 10% suspension of fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) that had been prewashed with 1% ovalbumin (Worthington)/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$/0.15 M NaCl/0.02 M Tris Cl, pH 7.2. Proteins were then solubilized by boiling for 5 min in 2% $NaDodSO_4$/10% glycerol/0.125 M Tris Cl, pH 6.7/5% 2-mercaptoethanol.

Total protein from labeled LSTRA or YAC-1 cells was solubilized after the cell pellets were washed with 10 ml of ice-cold 0.15 M NaCl/0.05 M Tris Cl, pH 7.5/0.01 M EDTA/0.01 M EGTA/0.01 M benzamidine (Sigma)/25 μM pepstatin A (Sigma)/1 mM diisopropyl fluorophosphate (Sigma)/0.01 M iodoacetate (Sigma)/1 μM myristate. The washed cells were lysed in 5% $NaDodSO_4$/10% glycerol/0.125 M Tris Cl, pH 6.7, and sonicated for 1 min with a Branson sonifier set at 1.2 A.

Samples were subjected to $NaDodSO_4$/PAGE according to Laemmli (23), with a stacking gel of 5% acrylamide and a 7–15% acrylamide linear-gradient separation gel, at 30 mA constant current. Molecular weight standards (Pharmacia) were bovine serum albumin (M_r 66,300), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,400), and α-lactalbumin (M_r 14,400), all tritiated by reductive methylation with NaB^3H_4 and formaldehyde (24). Samples were dissolved in 2% $NaDodSO_4$/10% glycerol/0.125 M Tris Cl, pH 6.7/5% 2-mercaptoethanol and boiled for 5 min immediately before electrophoresis. Proteins were fixed in the gels by gentle agitation with 500 ml of methanol/dimethylsulfoxide (30:70) for 10 min and then with 500 ml of dimethylsulfoxide for 1 hr. For fluorography, the gel was then treated with 200 ml of 22.2% 2,5-diphenyloxazole (Packard) in dimethylsulfoxide for 3 hr (25). The 2,5-diphenyloxazole was precipitated *in situ* by treating the gel with cold running tap water for 30 min. The gel was dried for 90 min in a Bio-Rad gel dryer, overlaid with Kodak X-Omat AR film, and stored at −76°C for 7–10 days.

For identification of fatty acids in proteins after $NaDodSO_4$/PAGE, regions of dried gels containing labeled protein were excised and soaked briefly in water to remove the paper backing. The gel pieces were then homogenized in 0.1% $NaDodSO_4$/0.05 M ammonium bicarbonate/5% 2-mercaptoethanol, heated at 100°C for 5 min, and then incubated at 37°C for 16 hr. The gel extract was filtered through a 0.22 μm Millex filter and lyophilized. A solution of egg-white lysozyme (0.2 ml, 10 mg/ml) was added and the proteins were precipitated by addition of 10 ml of acetone at 0°C. A mixture containing 20 nmol each of capric, lauric, myristic, palmitic, and stearic acids in chloroform was added and dried onto the protein precipitate. The protein was then hydrolyzed in 200 μl of 5.7 M HCl at 110°C for 20 hr *in vacuo*. Fatty acids were extracted in five successive 0.5-ml aliquots of chloroform. The chloroform extract was back-extracted with 0.5 ml of 1 mM HCl and then dried over anhydrous sodium sulfate, and the solvent was evaporated with a gentle nitrogen stream. The extracted fatty acids were dissolved in 0.1 ml of acetone and converted to phenacyl esters with a 2-fold molar excess of α-bromoacetophenone (Aldrich) and a 1-fold molar excess of triethylamine (Sigma) in acetone at 50°C for 2 hr (26). The phenacyl esters were resolved on an IBM octyl HPLC column. The mobile phase was acetonitrile/water (60:40, vol/vol), the flow rate was 2 ml/min, and the concentration of acetonitrile was increased linearly to 100% over 30 min.

RESULTS AND DISCUSSION

When the murine lymphoma cell line LSTRA was cultured in medium containing [^{3}H]myristate, label was incorporated into cellular proteins. In a typical experiment with one maxiplate of cells (≈4 × 10^7 cells), ≈50% of the label entered the cells in 4 hr and 0.4% of that (600,000 cpm) was found in the postnuclear particulate proteins. Upon $NaDodSO_4$/PAGE (gradient gel), several proteins appeared to be labeled (Fig. 1, lane D), the most striking of which had a M_r of 56,000. Other significant labeled components were of M_r 62,000 and 41,000. Of the several labeled proteins in the extract, only the major [^{3}H]myristate-labeled protein was recognized and precipitated by antibody (12) directed against the site of tyrosine phosphorylation in pp56, the prominent tyrosine protein kinase present in LSTRA cells (lanes E–H). The experiment included tests with different ratios of antibody solution to detergent extract, in order to determine optimal conditions for immunoprecipitation. When the antibody used in these

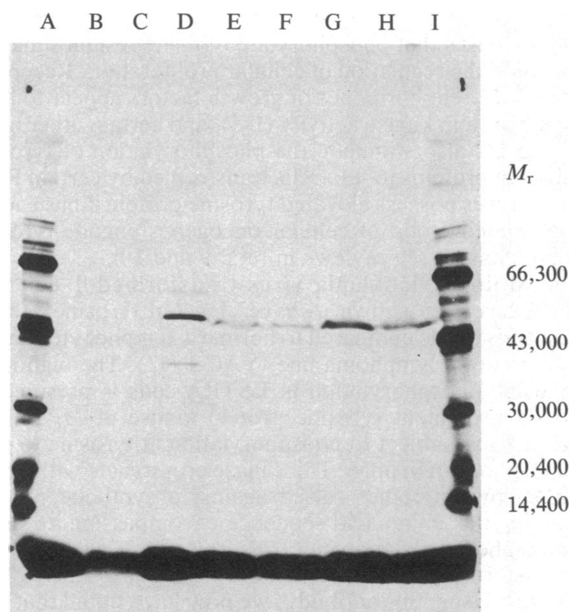

FIG. 1. Fluorograph after $NaDodSO_4$/PAGE showing [^{3}H]myristate- or [^{3}H]palmitate-labeled LSTRA cell postnuclear particulate fractions and immunoprecipitates from detergent extracts of these fractions. Palmitate-labeled particulate extract from ≈5 × 10^6 cells was either applied directly (lane B) or immunoprecipitated with 25 μl of antibody solution before application (lane C). Similarly, myristate-labeled particulate extract was either applied directly (lane D), or immunoprecipitated with either 25 μl (lane E) or 50 μl (lane F) of antibody solution. Lanes G and H are identical to lanes E and F except that twice as much extract (derived from ≈1 × 10^7 cells) was applied. Tritiated molecular weight standards are in lanes A and I.

experiments was pretreated with the peptide Lys-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly, which was used to evoke the antibody (12), immunoprecipitation of the labeled 56,000 M_r protein was prevented (Fig. 2, lane C as compared to lane B). This finding tended to rule out the possibility that the myristoylated 56,000 M_r protein was precipitated nonspecifically by the antibody and supported the concept that this protein is identical to pp56, the LSTRA tyrosine kinase. In contrast to the results obtained by exposing LSTRA cells to [^{3}H]myristate, similar treatment with [^{3}H]palmitate led to no significant retention of label in proteins after boiling with 2-mercaptoethanol prior to NaDodSO$_4$/PAGE (Fig. 1, lanes B and C).

Of the several labeled proteins other than pp56 seen in Fig. 1, it is probable on the basis of size that the protein of M_r 41,000 is the catalytic subunit of the cAMP-dependent protein kinase, the first protein shown to be modified by myristate (14). The identity of the protein of M_r 62,000, which does not interact with the antibody, is unknown. It is perhaps surprising that no labeled pp60$^{c\text{-}src}$ was found in these experiments, since it is known that pp60^{src} is myristoylated (B. Sefton, personal communication) and would be expected to react with the antibody employed here (12). Perhaps this protein was present at too low a level to be detected.

Since metabolic events could alter the nature of the labeled fatty acid in cell cultures, the chemical nature of the lipid(s) present in the immunoprecipitated, labeled 56,000 M_r band from the experiment of Fig. 1 was examined. For this, the labeled bands (lanes E–H) were excised, pooled, homogenized, extracted, acid hydrolyzed, and analyzed for fatty acids by HPLC of their phenacyl esters as described in *Materials and Methods*. As shown in Fig. 3, five added internal standard fatty acids were well resolved in this system. Ninety-six percent (12,173 cpm) of the applied radioactivity was eluted with the standard myristic acid. No radioactivity remained in the 5.7 M HCl phase, evidence against metabolic conversion of [^{3}H]myristate into acid-soluble hydrophilic compounds such as amino acids or sugars. The resistance of the pp56 protein-myristate linkage to boiling in 2% NaDodSO$_4$/10% glycerol/0.125 M Tris Cl, pH 6.7/5% 2-mercaptoethanol for 5 min is strongly suggestive of covalent attachment to pp56 and excludes (27) the thiol ester bond that characterizes more than 10 palmitoylated proteins (21, 28–32). Thus, it is clear that the attached group is myristate and that the attachment is covalent. In the catalytic subunit of cAMP-dependent protein kinase (14), the B chain of calcineurin (15), and Pr65^{gag} of Rauscher and Moloney murine leukemia viruses (16), single myristoyl groups have been shown to be in amide linkages with amino-terminal glycyl residues. The linkage of myristate to pp56 may be a fourth example, although evidence of the attachment site is not yet available. The possibility that [^{3}H]myristate might be present in pp56 as a result of adventitious noncovalent binding seems very remote inasmuch as the experiments carried out with [^{3}H]palmitate (Fig. 1, lanes B and C) show that long-chain fatty acids in general do not bind the protein nonspecifically in this type of experiment.

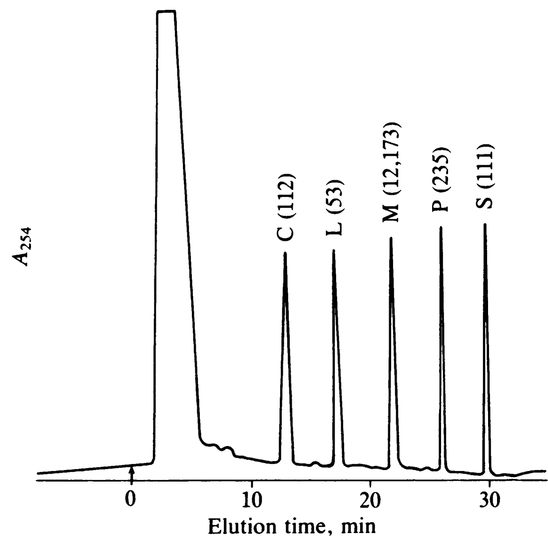

FIG. 3. HPLC of phenacyl esters of a mixture of a hydrolyzate of [^{3}H]myristate-labeled LSTRA pp56 and fatty acid standards. Gel pieces containing labeled LSTRA pp56 were excised from lanes E–H (Fig. 1), pooled, and extracted as described in *Materials and Methods*. The extract was mixed with 20 nmol each of capric, lauric, myristic, palmitic, and stearic acids (C, L, M, P, and S). After acid hydrolysis, the fatty acids were extracted, derivatized as described, and resolved by HPLC on an IBM octyl reversed-phase column at a flow rate of 2 ml/min with a linear gradient from 60 to 100% acetonitrile. The arrow indicates the time of injection of the sample, which contained 12,700 cpm. Fractions were monitored by absorbance at 254 nm and by scintillation counting in 20 ml of Aquasol. In parentheses above each internal standard fatty acid peak is the cpm recovered with that fatty acid; 96% of the extracted label was found in the derivatized myristate fraction.

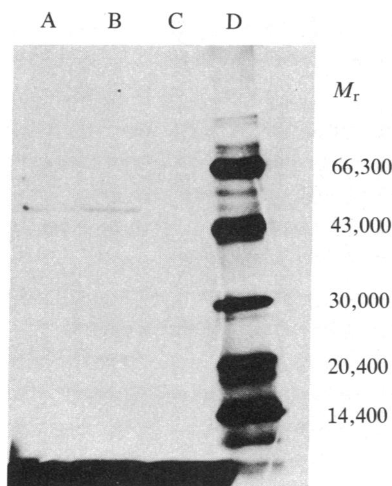

FIG. 2. Fluorograph after NaDodSO$_4$/PAGE showing the [^{3}H]myristate-labeled tyrosine protein kinase in immunoprecipitates of LSTRA extracts and inhibition of the immunoprecipitation by a peptide corresponding to the tyrosine phosphorylation site. Detergent extracts of 8 × 10^6 labeled LSTRA cells were incubated for 3 hr at 0°C with 50 μl (lane A) or 25 μl (lane B) of antibody solution or with 25 μl of antibody solution pretreated with 6 mM peptide containing the tyrosine phosphorylation site (lane C). Tritiated molecular weight standards are displayed in lane D. The film was exposed 10 days at −76°C.

YAC-1 is another murine lymphoma cell line derived from a mouse infected with the Moloney murine leukemia virus (11, 22). Incubation of YAC-1 cells with [^{3}H]myristate yielded a different pattern of labeling than that obtained with LSTRA cells (Fig. 4). The myristate-labeled proteins in extracts of YAC-1 cells are much more abundant than their counterparts in LSTRA cells, with the single exception of pp56, which predominates in LSTRA extracts. The post-nuclear particulate fraction of similar cultures of concanavalin A-stimulated bovine T lymphocytes also contains a myristate-labeled protein of the same mobility in NaDodSO$_4$/PAGE as pp56 (data not shown). Casnellie *et al.* (11) reported the presence in normal T lymphocytes of a phosphorylated protein with the characteristics of pp56. Swarup *et al.* (33) reported a tyrosine protein kinase activity of M_r 55,000 in rat spleen, which may be identical with LSTRA pp56. These data suggest that these myristoylated and phosphorylated proteins represent a single cellular gene product

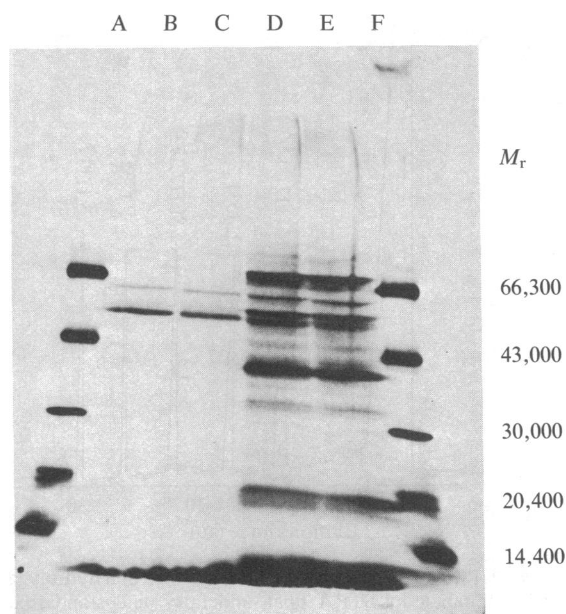

FIG. 4. Fluorograph after NaDodSO$_4$/PAGE of 5% NaDodSO$_4$ extracts ("total protein") of ≈4 × 10^7 LSTRA or YAC-1 murine lymphoma cells labeled for 4 hr with [^{3}H]myristate. Cell pellets were washed with a solution containing protease inhibitors, sonicated for 1 min in 5% NaDodSO$_4$/10% glycerol/0.125 M Tris Cl, pH 6.7, and subjected to NaDodSO$_4$/PAGE with (lane C, LSTRA; lane E, YAC-1) or without (lane B, LSTRA; lane D, YAC-1) treatment at 100°C with 5% 2-mercaptoethanol, pH 6.7, before electrophoresis. Lanes A and F contain tritiated molecular weight standards.

normally expressed at a low level in T lymphocytes. It will be interesting to see whether this protein is also myristoylated in normal spleen.

These observations add to the growing number of proteins that are reported to be conjugated to fatty acids. Although the nature and number of physiological roles of these hydrophobic elements are not clear, they have obvious potential for guiding interactions with membranes and other hydrophobic surfaces in cells. It remains to be seen whether the seemingly minor structural differences between myristate in amide linkage and palmitate in ester linkage mask important functional differences in the cellular environment, and whether the enzymes involved in regulation via phosphorylation and dephosphorylation utilize the myristoyl group in a common manner.

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