# Treatment of T Cells with 2-Hydroxymyristic Acid Inhibits the Myristoylation and Alters the Stability of p56$^{lck}$ †

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ABSTRACT: *N*-Myristoylation of p56$^{lck}$, a member of the Src family of protein-tyrosine kinases, is essential for its proper targeting to the plasma membrane. 2-Hydroxymyristic acid (HMA) is an analog of myristic acid that becomes metabolically activated in cells to form 2-hydroxymyristoyl-CoA, a potent inhibitor of myristoyl-CoA:protein *N*-myristoyltransferase (NMT), the enzyme that catalyzes protein *N*-myristoylation [Paige, L. A., Zheng, G.-q., DeFrees, S. A., Cassady, J. M., & Geahlen, R. L. (1990) *Biochemistry 29,* 10566]. In the presence of HMA, LSTRA cells, which overexpress p56$^{lck}$, synthesized nonmyristoylated p56$^{lck}$, which displayed a reduced electrophoretic mobility on SDS–polyacrylamide gels identical to that of a nonmyristoylated Gly$^2$ → Ala$^2$ mutant of p56$^{lck}$. Treatment with myristic acid, 2-hydroxypalmitic acid, or 2-fluoromyristic acid did not result in the synthesis of nonmyristoylated p56$^{lck}$. In contrast to the membrane-associated, myristoylated p56$^{lck}$, nonmyristoylated p56$^{lck}$ was cytosolic. Although nonmyristoylated p56$^{lck}$ retained tyrosine kinase activity, it was not labeled *in vivo* with [$^{32}$P]orthophosphate, indicating that a change in subcellular location altered its state of phosphorylation. A pulse-chase analysis revealed that cytosolic, nonmyristoylated p56$^{lck}$ was less stable than the myristoylated enzyme. In cell lines that do not overexpress p56$^{lck}$, HMA treatment resulted in a reduction in the levels of both newly synthesized and total p56$^{lck}$. Treatment of CD4$^+$ cells with HMA caused a corresponding decrease in the amount of CD4-associated p56$^{lck}$. Thus, chemical inhibition of protein *N*-myristoylation with HMA is an effective method for reducing the amount of p56$^{lck}$ available at the plasma membrane for signal transduction.

p56$^{lck}$ is a Src family protein-tyrosine kinase that is found predominantly in lymphoid cells. The structural features of p56$^{lck}$ are typical for the Src family and include (i) a conserved C-terminal kinase domain of approximately 250 amino acids containing two conserved tyrosine residues, Tyr 505 and Tyr 394; (ii) SH3 and SH2 domains located between amino acids 65 and 185; (iii) a unique N-terminal domain of 60 amino acids; (iv) and an N-terminal glycine residue that is the site for modification with myristic acid [reviewed in Sefton (1991)]. In T lymphocytes, p56$^{lck}$ is found bound to distinct cytoplasmic regions of the glycoprotein receptors, CD4 and CD8 (Rudd et al., 1988; Veillette et al., 1988a; Shaw et al., 1989, 1990; Turner et al., 1990) and exhibits altered tyrosine kinase activity when these receptors are cross-linked with antibodies (Veillette et al., 1989; Luo & Sefton, 1990). Thus, p56$^{lck}$ is involved in the early signaling events of these receptors.

Studies of the effects of *N*-myristoylation on the properties of p56$^{lck}$ have centered on the analysis of N-terminal mutants expressed in fibroblasts and HeLa cells. Such studies indicate that myristoylation is required for stable association with the plasma membrane as evidenced by the finding that non-myristoylated mutants of p56$^{lck}$ are predominantly cytosolic (Abraham et al., 1990). While myristoylation is not absolutely required for association with CD4, nonmyristoylated p56$^{lck}$ binds CD4 much less efficiently (Shaw et al., 1990). As is the case for pp60$^{v\text{-}src}$, a nonmyristoylated form of constitutively activated p56$^{lck}$ leads to a kinase that is transformation defective (Abraham et al., 1990). This nonmyristoylated p56$^{lck}$ is about 2.5-fold less active as a tyrosine kinase than its myristoylated counterpart and has decreased phosphorylation on tyrosine 394, the major site of autophosphorylation (Abraham et al., 1990). These transfection studies suggest that myristoylation is required both for the proper targeting and for full enzymatic activity of p56$^{lck}$.

In cells, 2-hydroxymyristic acid (HMA)[1] is metabolically converted to 2-hydroxymyristoyl-CoA, a potent inhibitor of myristoyl-CoA:protein *N*-myristoyltransferase (NMT) (Paige et al., 1990). The effects of HMA are specific to the inhibition of protein *N*-myristoylation catalyzed by NMT because other protein acylation events such as posttranslational palmitoylation are not altered. This raised the possibility that HMA could be used to inhibit the myristoylation of p56$^{lck}$ in cells that normally express the enzyme. We report here that treatment with HMA results in the production of nonmyristoylated p56$^{lck}$ in LSTRA cells, which normally overexpress the kinase. This nonmyristoylated p56$^{lck}$ displays a reduced mobility on SDS–PAGE gels and is cytosolic. Nonmyristoylated p56$^{lck}$ is turned over more rapidly than myristoylated p56$^{lck}$, and in cell lines that express normal levels of the kinase, HMA treatment results in a depletion of p56$^{lck}$.

## MATERIALS AND METHODS

### *Materials*

[$^3$H]Myristic acid (39.3 Ci/mmol), [$\gamma$-$^{32}$P]ATP (>7000 Ci/mmol), and [$^{32}$P]orthophosphate (8500–9120 Ci/mmol)

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[1] Abbreviations: NMT, myristoyl-CoA:protein *N*-myristoyltransferase; Tris, tris(hydroxymethyl)aminomethane; HMA, 2-hydroxymyristic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; PVDF, poly(vinylidene difluoride).

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were obtained from New England Nuclear. Tran-[$^{35}$S]-Label, a mixture of [$^{35}$S]methionine and [$^{35}$S]cysteine (>1000 Ci/mmol), was purchased from ICN. Protein A–Sepharose, bovine serum albumin, Celite, RPMI 1640, Grace's low-methionine media, and media supplements were purchased from Sigma. Protein G+–Sepharose was obtained from Oncogene Sciences. Phosphate-free RPMI was prepared from an RPMI 1640 select-amine kit purchased from Gibco. 2-Hydroxymyristic acid and 2-hydroxypalmitic acid were purchased from Lancaster, and myristic acid was purchased from Aldrich. 2-Fluoromyristic acid was synthesized as described previously (Paige et al., 1990). Production of polyclonal antipeptide antisera directed against a peptide corresponding to the 33 C-terminal amino acids of p56$^{lck}$ was prepared as previously described (Wang et al., 1991). Monoclonal anti-CD4 antibody was prepared from the GK1.5 mouse–rat hybridoma obtained from ATCC.

*Methods*

*Delivery of Fatty Acid Analogs to Cells.* The LSTRA and C10.9 murine T cell lines and Jurkat, a human T cell line, were all cultured as described previously (Paige et al., 1990). 2-Hydroxymyristic acid, 2-fluoromyristic acid, myristic acid, and 2-hydroxypalmitic acid were all delivered to cells in tissue culture as fatty acid–BSA complexes. Fatty acids were removed from Sigma fraction V BSA (Chen, 1967), and Celite was coated with fatty acid analogs as previously described (Spector & Hoak, 1969; Spector, 1986) except that 1 mmol of analog was added per gram of Celite. Fatty acid-coated Celite was then mixed with fatty acid-free BSA for 1 h at room temperature, after which time the Celite was removed by filtration with an Amicon 0.2-$\mu$m syringe filter. Fatty acid-coated BSA was added to cells at a final BSA concentration of 5 mg/mL containing approximately 1 mM HMA.

*Metabolic Labeling of p56$^{lck}$.* For immunoprecipitation of [$^{35}$S]methionine-labeled p56$^{lck}$, 3 × 10$^6$ LSTRA or Jurkat cells were labeled in 1 mL of low-methionine RPMI 1640 (1.5 mg/L) with 100–200 $\mu$Ci of Tran-[$^{35}$S]-Label for 5 h following a preincubation for 1 h with fatty acid-free BSA or BSA complexed with 2-hydroxymyristic acid, 2-fluoromyristic acid, myristic acid, or 2-hydroxypalmitic acid. For the pulse-chase analysis, LSTRA cells were treated and labeled as described but were then resuspended into 1 mL of RPMI 1640 with a normal level of methionine (15 mg/L) for 1, 5, or 9 h before harvesting. For immunoprecipitation of $^{32}$P-labeled p56$^{lck}$, LSTRA cells were preincubated with HMA–BSA for 44 h and then with phosphate-free RPMI 1640 for 4 h followed by addition of 1 mCi of [$^{32}$P]orthophosphate for 1 h.

Cells were pelleted and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 150 mM NaCl, and 10 $\mu$g/mL each of of leupeptin and aprotinin). Lysates were centrifuged at 2400$g$ for 10 min, and supernatants were equalized for protein and incubated for 1 h at 4 °C with anti-p56$^{lck}$ polyclonal antisera bound to protein A–Sepharose. In some experiments, the 33-residue peptide antigen (100 $\mu$M) was preincubated with the antisera prior to addition of the supernatant to competitively block the binding of the polyclonal antisera to p56$^{lck}$. The immune complexes were collected by centrifugation at 12000$g$ for 1 min and washed four times with buffer A (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 10 $\mu$g/mL each of leupeptin and aprotinin), four times with buffer A containing 2 M NaCl and 0.5% sodium deoxycholate, two times with phosphate-buffered saline containing 1% Triton X-100 and the protease inhibitor mixture, and two times with buffer containing 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, and 10 $\mu$g/mL each of leupeptin and aprotinin. Radiolabeled proteins were separated on 8% SDS–polyacrylamide gels (Laemmli, 1970) and visualized by fluorography (Soltysiak et al., 1984).

For analysis of myristoylated proteins, 3 × 10$^6$ LSTRA cells in 1 mL of RPMI 1640 were labeled with 50–100 $\mu$Ci of [$^3$H]myristate for 4 h following a preincubation with fatty acid-free BSA or BSA complexed with HMA for the times indicated. Cells were lysed in 5 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 2% Triton X-100, and 20 $\mu$g/mL each of leupeptin and aprotinin. Supernatants from a 2400$g$ centrifugation were equalized for protein and separated on a 10% SDS–polyacrylamide gel. Radiolabeled proteins were detected by fluorography.

*Expression of Murine lck cDNA in Sf9 Cells.* cDNA encoding wild-type (wt) murine *lck* cDNA, as the *Stu*I 1.5 kb fragment subcloned into the filled in *Eco*RI site of pGEM-3Z (Shaw et al., 1989), and cDNA encoding the Gly$^2$ → Ala$^2$ substituted myristoylation-defective mutant (myr$^-$) of murine *lck*, as the 1.5 kb *Xho*I to *Eco*RI fragment subcloned into Bluescript IISK (Shaw et al., 1990), were generously provided by Dr. Andrey Shaw, Washington University. The 1.5 kb *Eco*RI fragment representing the full-length wt *lck* cDNA was subcloned into the *Eco*RI site of the baculovirus transfer plasmid pVL1392 (Invitrogen). The full-length myr$^-$ *lck* cDNA, as the 1.5 kb *Xho*I to *Eco*RI fragment, was inserted first into pGEM11Zf (Promega), and then the 1.5 kb *Not*–*Eco*RI fragment was subcloned into the *Not*I and *Eco*RI cut pVL1392. The transfer plasmid constructs with the desired structure were identified, amplified in DH-5$\alpha$ *Escherichia coli*, and purified by the PEG method (Sambrook et al., 1989). These were cotransfected into Sf9 insect cells with AcMNPV viral DNA, and virus directing the expression of wt *lck* or myr$^-$ *lck* was isolated as described (Summers & Smith, 1987). Low-passage, high-titer stocks of each virus were used to infect (MOI greater than 10) individual 100-mm Petri dishes of 5 × 10$^7$ Sf9 cells per dish. Cells were harvested 18 h after infection by suspending in medium and sedimenting at 500$g$ for 10 min. Infected cells (3 × 10$^6$, wt or myr$^-$) were labeled with Tran-[$^{35}$S]-Label in low-methionine Grace's media for 5 h and immunoprecipitated as described above.

*Cell Fractionation.* To obtain soluble and particulate fractions, LSTRA cells were collected in swelling buffer (5 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 20 $\mu$g/mL each of leupeptin and aprotinin) and broken by 50 strokes of a dounce homogenizer. The particulate fraction was prepared by centrifugation at 200000$g$ for 1 h. The remaining supernatants were concentrated 10-fold in Centricon microconcentrators (Amicon, MW cutoff of 10 000). The soluble and particulate fractions were equalized for protein and were either analyzed for p56$^{lck}$ by immunoblotting or immunoprecipitation as described above.

*Immunoblotting for p56$^{lck}$.* Proteins present in lysates from LSTRA cells treated with HMA–BSA for 6, 24, or 48 h, were separated on an 8% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. Western blotting was performed as previously described with polyclonal antisera to p56$^{lck}$ (Wang et al., 1991).

*In Vitro Phosphorylation of p56$^{lck}$.* LSTRA cells (3 × 10$^5$/mL) were preincubated with HMA–BSA for 44 h and were fractionated as described above. Soluble and particulate fractions were equalized for protein, immunoprecipitated, and washed five times with lysis buffer. The immune complexes were incubated with a phosphorylation reaction mixture

containing 10 mM $MnCl_2$, 5 mM *p*-nitrophenyl phosphate, 20 mM Hepes, pH 7.5, and 20 $\mu$Ci [$\gamma$-$^{32}$P]ATP for 1 min at 30 °C. Phosphoproteins were separated on an 8% SDS–polyacrylamide gel, transferred to a poly(vinylidene difluoride) (PVDF) membrane, and detected by autoradiography. To selectively remove phosphate from phosphoserine and phosphothreonine residues, the PVDF membrane was immersed in 1 N KOH for 2 h at 58 °C.

*CD4 Cross-Linking and p56$^{lck}$ Activation.* C10.9 cells were treated with HMA–BSA for 48 h at $3 \times 10^5$ cells/mL; $3 \times 10^6$ cells were pelleted and resuspended in Brij lysis buffer (1% Brij 96, 150 mM NaCl, 25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM sodium orthovanadate, 200 $\mu$g/mL soybean trypsin inhibitor, and 20 $\mu$g/mL each of leupeptin and aprotinin). p56$^{lck}$ was immunoprecipitated with anti-CD4 antibody bound to protein G+–Sepharose as described above except that the immune complexes were washed four times with Brij lysis buffer and one time with a buffer containing 150 mM NaCl, 25 mM Hepes, pH 7.5, 1 mM sodium orthovanadate, and 20 $\mu$g/mL each of leupeptin and aprotinin. *In vitro* kinase reactions were performed in 10 mM $MnCl_2$, 5 mM *p*-nitrophenyl phosphate, 25 mM Hepes, pH 7.5, and 25 $\mu$Ci [$\gamma$-$^{32}$P]ATP for 1 min at 30 °C. Radiolabeled proteins were separated on a 10% SDS–polyacrylamide gel, transferred to a PVDF membrane, subjected to alkali hydrolysis in KOH, and detected by autoradiography.

## RESULTS

*Synthesis of Nonmyristoylated p56$^{lck}$ in LSTRA Cells.* 2-Hydroxymyristic acid was shown previously to inhibit protein *N*-myristoylation without affecting protein palmitoylation in cultured cells (Paige et al., 1990). As shown in Figure 1A, HMA inhibited protein *N*-myristoylation in LSTRA cells, a cultured murine T cell line that overproduces p56$^{lck}$ (Hurley & Sefton, 1989). To examine the effect of HMA on newly synthesized p56$^{lck}$, the protein was immunoprecipitated from [$^{35}$S]methionine-labeled cells that were preincubated with or without HMA. Treatment with HMA resulted in the production of a new form of p56$^{lck}$, which had a reduced electrophoretic mobility as assessed by SDS–PAGE (Figure 1B). Both of the radiolabeled protein bands were blocked from immunoprecipitation by a peptide that competitively bound the polyclonal antisera specific for p56$^{lck}$, indicating that both labeled proteins were forms of p56$^{lck}$ (Figure 1C). Consistent with this observation, both forms of p56$^{lck}$ yielded identical peptide maps following partial proteolysis, with *Staphylococcus aureus* V8 protease (data not shown).

Since HMA–CoA is an inhibitor of but not an alternate substrate for NMT (Paige et al., 1990), it was likely that the form of p56$^{lck}$ with reduced electrophoretic mobility represented a nonmyristoylated form of the enzyme. Indeed, p56$^{lck}$ synthesized in the presence of HMA had the same altered electrophoretic mobility as myr$^-$ p56$^{lck}$ expressed in Sf9 cells, which also migrated more slowly on SDS–polyacrylamide gels than the expressed, myristoylated form (Figure 2). Expression of a myr$^-$ p56$^{lck}$ in fibroblasts and a T cell hybridoma cell line also produces a form of the enzyme with a reduced electrophoretic mobility (Abraham & Veillette, 1990; Caron et al., 1992).

Several other fatty acid analogs were unable to cause the production of nonmyristoylated p56$^{lck}$. For these studies, LSTRA cells were preincubated with HMA, 2-fluoromyristic acid, myristic acid, or 2-hydroxypalmitic acid. p56$^{lck}$ was immunoprecipitated from cells labeled with [$^{35}$S]methionine and analyzed by SDS–PAGE. Only treatment with HMA resulted in the synthesis of p56$^{lck}$ with reduced electrophoretic mobility (data not shown).

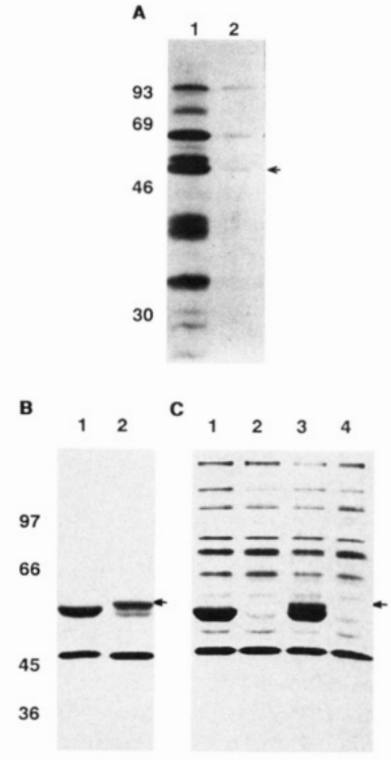


FIGURE 1: Synthesis of nonmyristoylated p56$^{lck}$ in the presence of HMA. (A) LSTRA cells pretreated with (lane 2) or without (lane 1) HMA were incubated with [$^3$H]myristate as described under Materials and Methods. Labeled proteins in cell lysates were separated by SDS–PAGE and visualized by fluorography. The migration position of p56$^{lck}$ is denoted with an arrow. (B) LSTRA cells pretreated with (lane 2) or without (lane 1) HMA were metabolically labeled with [$^{35}$S]methionine. p56$^{lck}$ was immunoprecipitated and visualized by fluorography following SDS–PAGE. The location of the slower migrating form of p56$^{lck}$ is denoted with an arrow. The migration positions of standard proteins of known molecular masses are indicated (in kilodaltons). (C) LSTRA cells were pretreated with (lanes 3 and 4) or without (lanes 1 and 2) HMA, and p56$^{lck}$ was immunoprecipitated as above except that a C-terminal 33 amino acid peptide which competitively bound the anti-p56$^{lck}$ antisera was added to the lysates in lanes 2 and 4. The arrow denotes nonmyristoylated p56$^{lck}$.

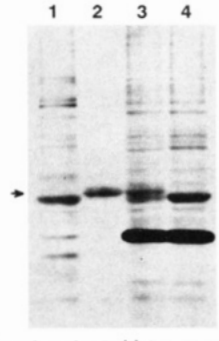


FIGURE 2: Nonmyristoylated p56$^{lck}$ has an altered electrophoretic mobility. p56$^{lck}$ was immunoprecipitated from [$^{35}$S]methionine-labeled Sf9 cells expressing wt (lane 1) or myr$^-$ (lane 2) p56$^{lck}$ and from [$^{35}$S]methionine-labeled LSTRA cells pretreated with (lane 3) or without HMA (lane 4). Radiolabeled proteins were separated by SDS–PAGE and visualized by fluorography. The arrow denotes nonmyristoylated p56$^{lck}$.

*Nonmyristoylated p56$^{lck}$ Accumulates over Time.* Since LSTRA cells produce large quantities of p56$^{lck}$, the HMA-induced production of nonmyristoylated p56$^{lck}$ over time could be assessed readily by immunoblotting. LSTRA cells were

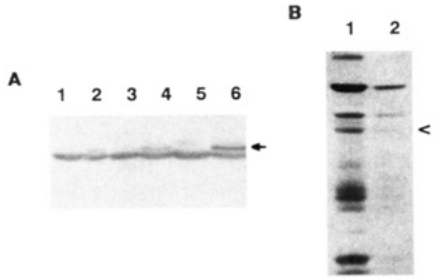


FIGURE 3: Accumulation of nonmyristoylated p56$^{lck}$ with time. (A) Proteins present in lysates from LSTRA cells untreated (lanes 1, 3, and 5) or treated with a single dose of HMA for 6, 24, and 48 h (lanes 2, 4, and 6) were separated by SDS–PAGE, transferred to nitrocellulose, and immunoblotted for p56$^{lck}$. The migration position of nonmyristoylated p56$^{lck}$ is indicated by the arrow. (B) Proteins present in lysates from LSTRA cells that were pretreated without (lane 1) or with a single dose of HMA (lane 2) for 44 h followed by metabolic labeling with [$^3$H]myristate for 4 h were separated by SDS–PAGE and detected by fluorography. The migration position of p56$^{lck}$ is indicated (<).

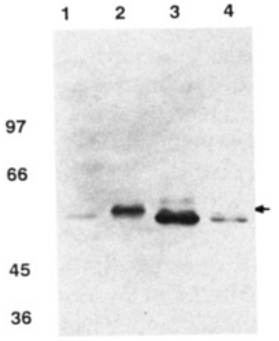


FIGURE 4: Nonmyristoylated p56$^{lck}$ is cytosolic. LSTRA cells, treated with (lanes 2 and 4) or without (lanes 1 and 3) HMA, were fractionated into soluble (lanes 1 and 2) or particulate fractions (lanes 3 and 4). Proteins were separated by SDS–PAGE and transferred to nitrocellulose. p56$^{lck}$ was detected by immunoblotting with anti-p56$^{lck}$ antibodies. The migration position of nonmyristoylated p56$^{lck}$ is indicated by the arrow.

treated with a single dose of HMA for 6, 24, or 48 h. Detergent lysates were analyzed for p56$^{lck}$ by immunoblotting. As shown in Figure 3A, the production of nonmyristoylated p56$^{lck}$ increased with time of treatment. After 48 h of treatment, a significant fraction of the total pool of p56$^{lck}$ in LSTRA cells consisted of nonmyristoylated enzyme. To verify that protein myristoylation was still inhibited 48 h following the addition of a single dose of HMA, treated and untreated cells were metabolically labeled for 4 h with [$^3$H]myristic acid 44 h following the addition of HMA. There was still a considerable reduction in the amount of [$^3$H]myristate incorporated into protein at this time for HMA-treated as compared to control cells (Figure 3B). Thus, the addition of a single dose of HMA, delivered as a HMA–BSA complex, leads to a prolonged inhibition of protein myristoylation in cultured cells.

*Nonmyristoylated p56$^{lck}$ Is Cytosolic, Active, and Not Phosphorylated.* LSTRA cells were treated with HMA for 48 h, lysed, and separated into soluble and particulate fractions. While myristoylated p56$^{lck}$ was found primarily in the particulate fraction, nonmyristoylated p56$^{lck}$ was predominantly cytosolic (Figure 4). This result is consistent with previous studies that have indicated that myristoylation is essential for the membrane localization of p56$^{lck}$ (Abraham & Veillette, 1990).

To characterize the activity of the cytosolic nonmyristoylated form of p56$^{lck}$, LSTRA cells were treated for 44 h with HMA

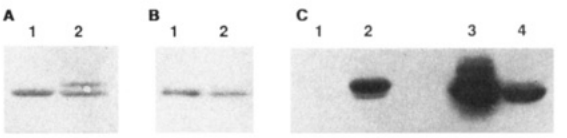


FIGURE 5: Nonmyristoylated p56$^{lck}$ has an altered state of phosphorylation. (A) LSTRA cells were pretreated for 44 h without (lane 1) or with (lane 2) HMA, and proteins present in lysates were separated by SDS–PAGE, transferred to nitrocellulose, and probed with anti-p56$^{lck}$ antibodies. Immunoreactive proteins were detected with a secondary antibody coupled to alkaline phosphatase. (B) In the same experiment, p56$^{lck}$ was immunoprecipitated from LSTRA cells labeled with [$^{32}$P]orthophosphate as described under Materials and Methods after 44 h of pretreatment with (lane 2) or without HMA (lane 1). Radiolabeled proteins were separated by SDS–PAGE and detected by autoradiography. (C) LSTRA cells treated with (lanes 2 and 4) or without (lanes 1 and 3) HMA were separated into soluble (lanes 1 and 2) and particulate (lanes 3 and 4) fractions. p56$^{lck}$ was immunoprecipitated from each fraction and assayed for kinase activity *in vitro*. The autophosphorylated kinase was visualized by autoradiography following SDS–PAGE.

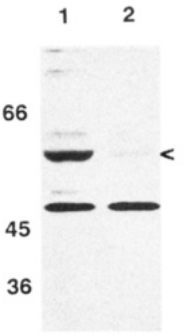


FIGURE 6: HMA causes a reduction of newly synthesized p56$^{lck}$ in Jurkat cells. Jurkat cells were pretreated with (lane 2) or without (lane 1) HMA prior to labeling with [$^{35}$S]methionine and immunoprecipitation of p56$^{lck}$ as described under Materials and Methods. The migration position of p56$^{lck}$ is indicated (<).

to allow the synthesis of the nonmyristoylated enzyme (Figure 5A). p56$^{lck}$ was then metabolically labeled with [$^{32}$P]orthophosphate (Figure 5B). Surprisingly, the cytosolic nonmyristoylated form of p56$^{lck}$ was not labeled with [$^{32}$P]orthophosphate. To rule out the possibility that the cytosolic nonmyristoylated p56$^{lck}$ was inactive, nonmyristoylated p56$^{lck}$ was immunoprecipitated from the soluble fractions of HMA-treated LSTRA cells and then incubated with [$\gamma$-$^{32}$P]ATP *in vitro* (Figure 5C). Since the cytosolic form of the kinase was still active, as shown by its ability to catalyze an autophosphorylation reaction, its *in vivo* phosphorylation state was most likely altered due to its change in subcellular location.

*HMA Treatment Results in a Net Decrease of Newly Synthesized p56$^{lck}$ in Cells That Do Not Overexpress p56$^{lck}$.* Inhibition of myristoylation by HMA in T cell lines that do not overexpress p56$^{lck}$ resulted in a net reduction in the level of newly synthesized p56$^{lck}$. In Jurkat cells, a human leukemia T cell line, pretreatment with HMA resulted in a reduced level of newly synthesized p56$^{lck}$ as determined by immunoprecipitation of the enzyme from [$^{35}$S]methionine-labeled cells (Figure 6). Similar results were seen with the T cell lines C10.9 and 2B4 (data not shown). The accumulation of nonmyristoylated p56$^{lck}$ was observed only in the p56$^{lck}$ overexpressing cell line LSTRA and not in any of the T cell lines that express more normal levels of the kinase.

To provide an explanation for the reduced level of p56$^{lck}$ observed in HMA-treated T cell lines, the rates of turnover of myristoylated and nonmyristoylated enzyme were compared in LSTRA cells. A pulse-chase analysis of metabolically labeled p56$^{lck}$ in HMA-treated LSTRA cells revealed that

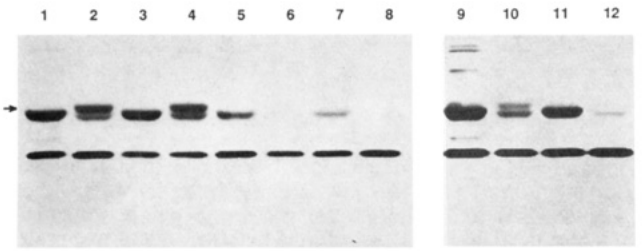


FIGURE 7: Pulse-chase analysis of myristoylated *vs* nonmyristoylated p56$^{lck}$ in LSTRA cells. LSTRA cells pretreated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) HMA were labeled with [$^{35}$S]methionine for 5 h and then transferred to fresh media without label for 0 (lanes 1 and 2), 1 (lanes 3 and 4), 5 (lanes 5 and 6), and 9 h (lanes 7 and 8). Radiolabeled p56$^{lck}$ was immunoprecipitated and detected by fluorography following SDS–PAGE. Lanes 9–12 represent a longer exposure to X-ray film of lanes 5–8 to allow visualization of radiolabeled bands from HMA-treated cells. The migration position of nonmyristoylated p56$^{lck}$ is indicated with an arrow.

the form of p56$^{lck}$ with a reduced electrophoretic mobility was degraded at a faster rate than was the normal myristoylated p56$^{lck}$. This indicated that the cytosolic nonmyristoylated p56$^{lck}$ was less stable than the membrane-associated myristoylated p56$^{lck}$ in LSTRA cells (Figure 7). Most likely, nonmyristoylated p56$^{lck}$ does not accumulate in cell lines that do not overexpress p56$^{lck}$ because it is degraded at a rapid rate.

*HMA Treatment of CD4$^+$ T Cells.* In T cells, p56$^{lck}$ is associated with the transmembrane MHC II receptor, CD4. Cross-linking CD4 results in the activation of receptor-associated p56$^{lck}$ (Veillette & Bookman, 1989; Luo & Sefton, 1990). A reduction in the level of p56$^{lck}$ should, therefore, result in a reduction in CD4-associated enzyme. To test this hypothesis, the CD4$^+$ T cell line C10.9 was treated with HMA for 48 h. The level of receptor-associated p56$^{lck}$ was assessed by immunoprecipitation with an anti-CD4 antibody (GK1.5) followed by autophosphorylation of the kinase in the immune complex. Treatment with HMA resulted in a decreased amount of p56$^{lck}$ associated with the CD4 receptor, which correlated with the decreased amount of total cellular p56$^{lck}$ (data not shown).

## DISCUSSION

We have shown previously that HMA is a selective inhibitor of protein *N*-myristoylation in LSTRA cells (Paige et al., 1990). HMA is metabolically activated by cells to form 2-hydroxymyristoyl-CoA, which is a potent inhibitor of NMT (Paige et al., 1990). This observation suggested the possibility that HMA could be used as a tool to examine the properties of myristoylated enzymes in cultured cells. In this investigation we sought to characterize the effects of HMA on the properties of a specific enzyme, the protein–tyrosine kinase p56$^{lck}$.

Because p56$^{lck}$ is overexpressed approximately 40-fold in LSTRA cells (Hurley & Sefton, 1989), this cell line was used as a model system. HMA treatment of LSTRA cells resulted in the production of an altered form of p56$^{lck}$ that displayed a reduced electrophoretic mobility on SDS–polyacrylamide gels. This altered mobility of p56$^{lck}$ is consistent with the synthesis of a nonmyristoylated form of the enzyme since a nonmyristoylated Gly$^2$ → Ala$^2$ mutant of p56$^{lck}$ also displayed the same altered electrophoretic mobility (Figure 2, Abraham et al., 1990; Caron et al., 1992). The identification of a nonmyristoylated form of p56$^{lck}$ is also consistent with its cytosolic localization (Figure 4) and the known mechanism of action of HMA. These results indicate that protein *N*-myristoylation, which occurs solely as a cotranslational process, is not an obligatory event required for the synthesis of proteins bearing NMT recognition sequences. Inhibition of NMT appeared to have little or no effect on the rate of synthesis of p56$^{lck}$.

The ability of HMA to inhibit myristoylation and thus induce the production of nonmyristoylated p56$^{lck}$ was a specific property of HMA since other related analogs, 2-fluoromyristic acid and 2-hydroxypalmitic acid, did not have similar effects (data not shown). Even though 2-fluoromyristic acid (FMA) is converted to 2-fluoromyristoyl-CoA, an inhibitor of protein *N*-myristoylation (Paige et al., 1990), it was not effective in causing the production of nonmyristoylated p56$^{lck}$ in LSTRA cells. This is likely due to the fact that 2-fluoromyristoyl-CoA is a less potent inhibitor of *N*-myristoylation than is 2-hydroxymyristoyl-CoA ($K_i$ of 200 nM *vs* 45 nM) and can serve as an alternate substrate for NMT (Paige et al., 1990). Metabolic labeling studies with 2-fluoro[$^3$H]myristic acid have confirmed that 2-fluoromyristic acid can be transferred to cellular target proteins in place of myristic acid (data not shown). 2-Hydroxymyristoyl-CoA is an inhibitor of NMT and does not serve as an alternative substrate as is evidenced by the fact that 2-hydroxymyristate is not transferred by NMT to a target peptide sequence (Paige et al., 1990). Treatment of cells with 2-hydroxypalmitic acid did not result in the production of nonmyristoylated p56$^{lck}$ as expected, since this compound is not an inhibitor of protein *N*-myristoylation (data not shown).

When HMA is delivered as a fatty acid–BSA complex to LSTRA cells, the inhibition of myristoylation persists over 48 h (Figure 3B), and the production of nonmyristoylated p56$^{lck}$ increases with time (Figure 3A). The increased production of nonmyristoylated p56$^{lck}$ after 48 h of HMA treatment of LSTRA cells allowed a further characterization of nonmyristoylated p56$^{lck}$. Cell fractionation studies revealed that nonmyristoylated p56$^{lck}$ was predominantly cytosolic (Figure 4). This is again consistent with the mutant Gly$^2$ → Ala$^2$ nonmyristoylated form of p56$^{lck}$ expressed in fibroblasts, which is also cytosolic (Abraham & Veillette, 1990), and underscores the importance of *N*-myristoylation to the membrane-localization of p56$^{lck}$.

p56$^{lck}$ contains two primary sites of tyrosine phosphorylation, Tyr$^{394}$, the site at which autophosphorylation occurs, and Tyr$^{505}$, a phosphorylation site that negatively regulates the activity of the kinase. For the nonmyristoylated form of p56$^{lck}$, neither of these sites was phosphorylated *in vivo*. The slower migrating form of p56$^{lck}$ was not labeled in cells incubated with [$^{32}$P]orthophosphate (Figure 5B) and did not react with antiphosphotyrosine antibodies on Western blots (data not shown). Others have reported decreases in the phosphorylation of Tyr$^{394}$ in nonmyristoylated mutants of p56$^{lck}$ (Abraham & Veillette, 1990) or the equivalent site in nonmyristoylated pp60$^{c\text{-}src}$ (Bagrodia et al., 1993) but generally have observed phosphorylation at the negative regulatory sites. The decreased phosphate content of nonmyristoylated p56$^{lck}$ could not be accounted for by a loss of activity since the enzyme, isolated from the cytosols of HMA-treated LSTRA cells, could readily catalyze an autophosphorylation reaction *in vitro* (Figure 5C). The most likely explanation for these observations is that in LSTRA cells a change in subcellular localization of p56$^{lck}$ exposes it to the actions of cytoplasmic phosphatases that dephosphorylate both primary sites of tyrosine phosphorylation.

p56$^{lck}$ can also be phosphorylated on serine and threonine residues *in vivo*. Hyperphosphorylation of p56$^{lck}$ resulting

from the treatment of cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an activator of protein kinase C, results in a form of p56$^{lck}$ with a reduced mobility on SDS–polyacrylamide gels (Casnellie & Lamberts, 1986; Veillette et al., 1988b). The reduced mobility of p56$^{lck}$ resulting from the treatment of cells with HMA was not due to hyperphosphorylation of p56$^{lck}$ since treatment of immune complexes from HMA-treated cells with potato acid phosphatase did not alter the mobility of the slower migrating form of p56$^{lck}$ (data not shown) and p56$^{lck}$ with reduced mobility was not labeled in cells incubated with [$^{32}$P]orthophosphate (Figure 5B).

Loss of the *N*-myristoyl group leads to a decrease in the stability of p56$^{lck}$. This effect is most obvious in T cells that express normal levels of the kinase where treatment of cells with HMA leads to a substantial decrease in the level of the kinase (Figure 6). In LSTRA cells, where the high level of expression of p56$^{lck}$ is driven by a strong retroviral promoter inserted upstream of the *lck* gene, sufficient synthesis occurs to allow accumulation of nonmyristoylated enzyme. Even in these cells, the nonmyristoylated enzyme is less stable than the myristoylated kinase as determined by a pulse-chase analysis (Figure 7). Since inhibition of *N*-myristoylation might be expected to yield a protein with an exposed amino terminus, we also examined the possibility that the slower migrating form of p56$^{lck}$ arose due to enzyme ubiquitination. Western blotting and immunoprecipitation experiments with anti-ubiquitin antibodies indicated no changes in cellular ubiquitination patterns and no addition of ubiquitin to p56$^{lck}$ in HMA-treated cells (data not shown). Thus, the increased rate of enzyme turnover is most likely a function of either an altered subcellular location or a reduced stability of the nonmyristoylated enzyme. For the catalytic subunit of the cAMP-dependent protein kinase, *N*-myristoylation is important not for membrane localization of the enzyme but for enhanced structural stability (Yonemoto et al., 1993).

p56$^{lck}$ normally associates with the cell surface glycoprotein CD4 and is thought to play a major role in signaling through this receptor. Nonmyristoylated p56$^{lck}$ is unable to stably associate with the plasma membrane, whereas it still associates to a small extent with CD4 when both proteins are coexpressed in HeLa cells (Shaw et al., 1990). We were interested in determining if the effects of HMA on p56$^{lck}$ were significant enough to alter the amount of p56$^{lck}$ associated with CD4. Treatment of CD4$^+$ C10.9 cells for 48 h resulted in a net decrease in the amount of p56$^{lck}$ associated with CD4 (data not shown). The nonmyristoylated form of p56$^{lck}$ had reduced stability in CD4$^+$ as well as CD4$^-$ cell lines, and no nonmyristoylated enzyme coprecipitated from C10.9 cells with CD4, indicating that the expression of CD4 does not compensate for the reduced stability of p56$^{lck}$. Therefore, HMA can be used to inhibit myristoylation, resulting in a net decrease in p56$^{lck}$ available for signaling. In preliminary experiments, we have found also that treatment of B cell lines with HMA results in a reduction in the levels of the myristoylated protein-tyrosine kinase, p53/56$^{lyn}$ (unpublished observations). Thus, HMA should prove to be a useful tool for studying the role of myristoylated kinases in signal transduction in a variety of cell types by causing the specific cellular depletion of these acylated enzymes.

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