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Myristic Acid Is Attached to the Transforming Protein of Rous Sarcoma Virus During or Immediately After Synthesis and Is Present in Both Soluble and Membrane-Bound Forms of the Protein

JANICE E. BUSS,* MARK P. KAMPS, AND BARTHOLOMEW M. SEFTON
Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138

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Myristic acid, a minor component of cellular fatty acids, has been shown previously to be covalently bound to most molecules of $p60^{src}$, the transforming protein of Rous sarcoma virus. We have now determined at what time during the life cycle of $p60^{src}$, and where within the cell, this lipid becomes attached to the protein. $p60^{src}$ was found to acquire myristic acid at only one time, during or immediately after its synthesis. $p60^{src}$ is known to be synthesized on free polysomes and appears at the cytoplasmic face of the plasma membrane after a lag of 10 min. The addition of myristic acid to $p60^{src}$ therefore precedes the binding of the protein to the plasma membrane. The lipid attached to $p60^{src}$ is a permanent, metabolically stable part of the protein; we found no evidence for turnover of the myristyl moiety. However, we did find myristate attached to various soluble forms of $p60^{src}$ and to a large number of cytosolic cellular proteins as well. This demonstrates that the attachment of myristic acid to a protein is not in itself sufficient to convert a soluble protein into a membrane-bound protein.

$p60^{src}$, the transforming protein of Rous sarcoma virus (RSV) (5) is a protein kinase which phosphorylates tyrosine residues in substrate proteins (7, 16, 22). $p60^{src}$ undergoes an unusual form of protein modification: the 14-carbon saturated fatty acid, myristic acid, is covalently attached to its amino terminus (39; J. E. Buss and B. M. Sefton, J. Virol., in press). In these studies we have characterized the time and intracellular location of myristic acid addition to $p60^{src}$ and analyzed the effect of temperature-sensitive mutations on this process.

Although few studies have examined the biochemistry of fatty acylation of proteins, two different types of modification are known to occur. The 16-carbon saturated fatty acid, palmitic acid, has been shown to be present in a variety of membrane-associated proteins. These include the transferrin receptor (24); $p21^{ras}$, the transforming protein of Harvey murine sarcoma virus (39); and the E1 and E2 glycoproteins of Sindbis virus and the G glycoprotein of vesicular stomatitis virus (31, 32). The palmityl group is attached to these proteins via ester bonds to cysteine (30) or possibly serine residues (32). The addition of ester-linked palmitate is clearly a posttranslational event. The fatty acid is added to viral glycoproteins in the Golgi apparatus ca. 20 min after their synthesis (33), and only the mature forms of $p21^{ras}$ and the transferrin receptor contain palmitic acid (25, 39).

Only five identified proteins are known to contain a covalently attached myristic acid. These are the cellular and viral forms of $p60^{src}$ (Buss and Sefton, in press), the catalytic subunit of the cyclic AMP-dependent protein kinase (6), the protein phosphatase, calcineurin B (1), the T-cell-specific p56 protein from LSTRA cells (41), and proteins which contain the $p15^{gag}$ protein of mammalian retroviruses (15, 35). This last group includes the unglycosylated forms of a number of *gag-onc* fusion proteins such as Abelson murine leukemia virus $P120^{gag\text{-}abl}$ and Snyder-Theilen feline sarcoma virus $P85^{gag\text{-}fes}$ (34). Where it has been studied, the myristyl group appears to be attached through an amide bond to the alpha-amino group of an N-terminal glycine residue. It is not known at what time during the life span of the polypeptide or where within the cell myristic acid becomes attached to these proteins.

Shortly after synthesis on free polyribosomes (21, 29), $p60^{src}$ forms a complex with two cellular proteins (3, 4, 8): a 90,000-dalton phosphoprotein (hsp90) whose synthesis is increased by stress (26) and a 50,000-dalton phosphoprotein (p50) which contains both phosphoserine and phosphotyrosine (2, 3, 14, 16, 27). Courtneidge and Bishop (8) and Brugge et al. (4) have suggested that this complex may act as a system to transport newly synthesized $p60^{src}$ to the plasma membrane where the majority of the $p60^{src}$ molecules reside (9, 19, 20). We have examined how soon after synthesis the myristyl group is added to $p60^{src}$ in two ways. First, we have determined whether the $p60^{src}$ found in the complex with hsp90 and p50 contains myristic acid. Second, we have measured the effect of the inhibition of protein synthesis on the incorporation of [^{3}H]myristic acid into the protein. We also have investigated the half-life of the myristyl moiety of $p60^{src}$ to determine whether myristylation is a reversible phenomenon.

The amount of lipid in the temperature-sensitive $p60^{src}$ protein of *ts*NY68 has been reported to be decreased significantly at the nonpermissive temperature (13). These studies, however, used [^{3}H]palmitic acid to quantify the amount of fatty acid in $p60^{src}$. Since all of the ^{3}H incorporation into $p60^{src}$ from [^{3}H]palmitic acid appears to result from catabolism of the palmitate to myristic acid (Buss and Sefton, in press), we have reexamined the effect of temperature-sensitive mutations on the addition of lipid to $p60^{src}$ with [^{3}H]myristic acid as the biosynthetic precursor.

We initially suggested that the lipid bound to $p60^{src}$ might help anchor the protein to the cytoplasmic face of the plasma membrane (39). This idea was supported by the report that the $p60^{src}$ proteins encoded by two variants of RSV both bound to membranes poorly and lacked lipid (13). Here we examine this idea further by determining the distribution between the cytoplasm and cellular membranes of lipid-modified $p60^{src}$ and a large spectrum of other fatty-acylated cellular proteins. We find that many myristylated proteins, including a subpopulation of $p60^{src}$, behave as soluble proteins during traditional cell fractionation. Attachment of

* Corresponding author.

myristic acid to a protein thus does not induce an irreversible association of the protein with cell membranes.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo cell cultures were prepared and infected with Schmidt-Ruppin RSV, subgroup D (SR-RSV-D); with SR-RSV, subgroup A (SR-RSV-A); with a mutant virus derived from SR-RSV-A, *ts*NY68 (17), which is temperature sensitive for transformation; or with a temperature-sensitive mutant of Prague RSV-A, *ts*LA29 (42), as described previously (37). Cells infected with SR-RSV-D were maintained at 41°C and subcultured the day before use in the Dulbecco-Vogt modification of Eagle medium supplemented with 2% tryptose phosphate broth and 4% calf serum. Cells infected with SR-RSV-A, *ts*NY68, or *ts*LA29 were maintained at 37°C for 3 days, at which time ca. 50% of the wild-type RSV-infected cells were morphologically transformed. Infected cells were then subcultured and allowed to grow for 2 more days at either 35 or 41°C in the same medium as the cells infected with SR-RSV-D.

Labeling with [^{3}H]palmitic acid, [^{3}H]myristic acid, and [^{35}S]methionine. 9,10-[^{3}H]palmitic acid (23.5 Ci/mmol; New England Nuclear Corp.) in toluene or 9,10-[^{3}H]myristic acid (12.9 Ci/mmol, New England Nuclear Corp.) in ethanol was dried and dissolved in dimethyl sulfoxide as described previously (39). The complete medium for labeling with fatty acids consisted of the Dulbecco-Vogt modified Eagle medium, 10% calf serum, 5 mM sodium pyruvate, nonessential amino acids, 1% dimethyl sulfoxide, and the indicated amounts of fatty acid. Cells were labeled with 0.1 mCi of [^{35}S]methionine per ml in methionine-free Dulbecco-Vogt modified Eagle medium containing 10% dialyzed calf serum. Labeling was for 2 h at 41°C in the case of SR-RSV-D-infected cells or for 2 h at 35 or 41°C as indicated in the case of cells infected with SR-RSV-A or mutant viruses.

Treatment with cycloheximide. Cells were treated with cycloheximide by replacement of their growth medium with fresh medium containing 20 μg of cycloheximide per ml. Control cultures also received fresh medium. The treated cells were then labeled for 2 h in radioactive medium which contained cycloheximide.

Immunoprecipitation and SDS-PAGE. Lysis of cells in RIPA buffer and immunoprecipitation with rabbit anti-RSV tumor sera were as described previously (36). For analysis of total cell protein, cultures labeled with [^{3}H]myristic acid or [^{3}H]palmitic acid were dissolved by adding electrophoresis sample buffer directly to a washed cell monolayer. Samples were transferred to tubes, boiled for 1 min, and then passed through a 27-gauge needle to shear cellular DNA. All samples were analyzed by electrophoresis on 15% polyacrylamide gels (37).

Sedimentation analysis of p60^{src}. For glycerol gradient analysis, 10^6 cells were lysed in 200 μl of Nonidet P-40 (NP-40) buffer (37) (1% NP-40, 0.15 M NaCl, 10 mM sodium phosphate [pH 7.2], 2 mM EDTA, 1% Trasylol). The lysate was clarified by centrifugation at 20,000 × *g* for 30 min and layered onto a 2.4-ml gradient of 5 to 20% glycerol in NP-40 buffer formed on top of 0.25 ml of 50% glycerol in NP-40 buffer. Centrifugation was for 19 h at 40,000 rpm (150,000 × *g*) at 4°C in a Beckman SW50.1 rotor. Gradient fractions (0.25 ml) were collected by puncturing the bottom of the tube.

Cell fractionation. All steps were carried out at 4°C. Metabolically labeled cells were swollen in hypotonic buffer containing 5 mM KCl, 1 mM $MgCl_2$, and 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0) and broken with 30 strokes in a tight-fitting Dounce homogenizer (9). The lysate was adjusted to 0.1 M NaCl (19), and soluble and particulate fractions were prepared directly from the cell lysate by centrifugation in Beckman Ultraclear tubes at 100,000 × *g* for 30 min at 4°C.

Fluorography and quantification of radioactive proteins. Polyacrylamide gels were impregnated with diphenyloxazole, dried, and exposed to presensitized X-ray film at −70°C. The relative amount of p60^{src} in separate lanes of the gel was quantified by scanning films with a Quick-Scan densitometer (Helena Laboratories) and calculating peak areas from the integrator tracings. For proteins labeled with [^{3}H]palmitic acid in the presence or absence of cycloheximide, scans of entire gel channels were cut from the chart paper and weighed. [^{3}H]myristic acid and [^{35}S]methionine incorporation into p60^{src} from *ts*NY68 cells was determined by excising pieces of the dried, diphenyloxazole-impregnated polyacrylamide gel and counting them directly in 3a70B scintillation fluid (Research Products International Corp.).

RESULTS

p60^{src} in the complex with hsp90 and p50 cellular proteins contains myristic acid. Chicken cells transformed with SR-RSV-A and grown at 41°C were labeled for 2 h with either [^{3}H]myristic acid or [^{35}S]methionine and fractionated by glycerol gradient sedimentation. p60^{src} was recovered from each gradient fraction by immunoprecipitation (Fig. 1). A total of 65% of the [^{35}S]methionine-labeled p60^{src} sedimented as a monomer (fractions 12 to 14); 35% was present in a more rapidly sedimenting complex with hsp90 and p50 (fractions 7 to 8). [^{3}H]myristic acid-labeled p60^{src} isolated by immunoprecipitation was distributed in exactly the same manner: 69% as a monomer and 31% in the complex. Neither hsp90 nor p50 contained myristate. Immunoprecipitation with this rabbit anti-RSV tumor serum detects ~25% of the p60^{src} present in a cell lysate (Buss and Sefton, in press). So few proteins contain myristic acid that p60^{src} also can be identified directly in cell lysates without immunoprecipitation (Buss and Sefton, in press). To ensure that we were not looking at a restricted subpopulation of p60^{src}, we also examined the sedimentation properties of the total population of [^{3}H]myristic acid-labeled p60^{src} directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A total of 61% of the [^{3}H]myristic acid-labeled p60^{src} sedimented as a monomer, and 39% was found in the high-molecular-weight complex. Both procedures thus show that the soluble, complex-bound form of p60^{src} contains myristic acid and suggest that myristylation of p60^{src} occurs during or before formation of the complex.

Addition of myristic acid to p60^{src} occurs rapidly and requires continuing protein synthesis. Because newly synthesized p60^{src} is found exclusively in the soluble complex with hsp90 and p50 and only later associates with cellular membranes, the presence of a myristyl group attached to p60^{src} in the complex raised the possibility that myristic acid was added to the protein before its initial interaction with membranes. The time of addition of myristic acid to p60^{src} was therefore examined by a second method. We inhibited the synthesis of p60^{src} with cycloheximide for 20 min and then labeled the treated cells with [^{3}H]myristic acid. In the presence of cycloheximide, incorporation of [^{3}H]myristic acid into p60^{src}, and into almost all other cellular proteins, was decreased to less than 1% of the incorporation in

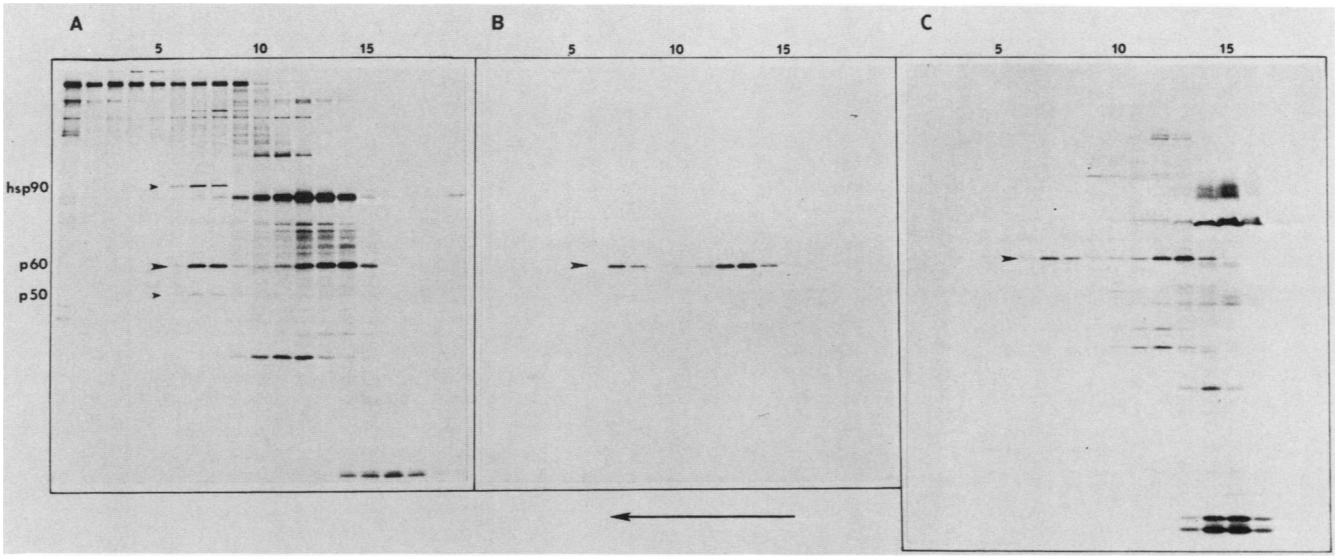

FIG. 1. [^{3}H]myristic acid-labeled p60^{src} is present in the complex with hsp90 and p50. SR-RSV-A-infected chicken cells were labeled for 2 h with [^{35}S]methionine or [^{3}H]myristic acid. Cells were lysed in NP-40 buffer, and the lysates were sedimented at 150,000 × *g* for 19 h at 4°C in gradients of 5 to 20% glycerol in NP-40 buffer. Proteins immunoprecipitated from gradient fractions with antitumor serum were analyzed by SDS-PAGE and visualized by fluorography. Gradient fractions from the [^{3}H]myristic acid-labeled cells were also analyzed by SDS-PAGE without immunoprecipitation. Exposure time was 6 h for [^{35}S]methionine-labeled proteins, 4 days for [^{3}H]myristic acid-labeled immunoprecipitated proteins, and 12 days for total [^{3}H]myristic acid-labeled proteins. hsp90 and p50 are indicated with arrows. The direction of sedimentation was from right to left and is indicated at the bottom of the figure. Fraction numbers are indicated at the top of the fluorograms. A, Immunoprecipitated proteins from [^{35}S]methionine-labeled cells; B, immunoprecipitated proteins from [^{3}H]myristic acid-labeled cells; C, total [^{3}H]myristic acid-labeled proteins.

untreated cells (Fig. 2). The lack of protein myristylation in the presence of cycloheximide was not due to inhibition of uptake or activation of myristic acid, for the amount of [^{3}H]myristic acid incorporated into phosphatidylcholine was the same for treated and control cultures (data not shown). To measure more precisely how soon myristylation occurs after the release of p60^{src} from the polysome, we decreased the time of pretreatment of the cells with cycloheximide. Even when cycloheximide and [^{3}H]myristic acid were added to the cells simultaneously, the incorporation of [^{3}H]myristic acid into p60^{src} and all but three other cellular proteins was abolished (Fig. 2). An immediate cessation of myristate incorporation into protein also occurred in cells treated with emetine (data not shown). p60^{src} molecules synthesized before the addition of the cycloheximide did not incorporate [^{3}H]myristic acid. Myristylation thus appears to occur only during or immediately after synthesis of the polypeptide for p60^{src} and the large majority of cellular myristic acid-containing proteins.

In contrast to the nearly total inhibition of protein myristylation, the attachment of palmitic acid to cellular proteins was less affected by cycloheximide (Fig. 2). Densitometric analysis indicated that exposure to cycloheximide for 20 min decreased the total incorporation of [^{3}H]palmitic acid into the cellular proteins resolved on our gels by 75%. The labeling of at least three minor proteins with molecular weights between 30,000 and 46,000 was unaffected by cycloheximide. When [^{3}H]palmitic acid was added simultaneously with emetine, only a 26% decrease in the incorporation of [^{3}H]palmitic acid was observed (data not shown).

The myristyl group of p60^{src} is metabolically stable. The lack of incorporation of myristic acid into p60^{src} molecules synthesized before the addition of cycloheximide suggested that if the myristyl moiety attached to p60^{src} was lost, it was not replaced. To examine the stability of the myristyl group of p60^{src} in another way, we measured the half-life of [^{3}H]myristic acid-labeled p60^{src}. Cells were labeled with [^{3}H]myristic acid or [^{35}S]methionine for 2 h and then incubated in nonradioactive medium for 3, 6, 10, or 22 h (Fig. 3). The amount of [^{35}S]methionine-labeled p60^{src} decreased by 24% in 3 h and by 98% in 22 h. The polypeptide chain of p60^{src} was therefore degraded with a half-life of ca. 7 h, in agreement with previous results (38). The amount of [^{3}H]myristic acid-labeled p60^{src} decreased by 28% in 3 h and by more than 95% in 22 h. The half-life of the myristate in p60^{src} thus appeared to be the same as the half-life of the protein itself. Because it is difficult to dilute the intracellular pool of radioactive lipids rapidly, continued incorporation of [^{3}H]myristic acid during the chase could potentially mask the loss of the myristyl moiety. Because the amount of [^{3}H]myristic acid in p60^{src} did, however, decrease substantially within the first 3 h, the extent of continued incorporation of [^{3}H]myristic acid after initiation of the chase would appear to be small. The myristyl group is therefore as metabolically stable as the polypeptide backbone of the p60^{src} protein.

Soluble cytoplasmic proteins can contain myristic acid. Both newly synthesized p60^{src} and the population of p60^{src} in the complex with hsp90 and p50 are reported to be cytosolic and, as shown above, to contain myristic acid. This suggested that it should also be possible to detect, by conventional cell fractionation procedures, soluble forms of p60^{src} which contain myristic acid. This experiment is difficult to perform quantitatively because with most strains of RSV less than 10% of p60^{src} behaves as a cytosolic protein during cell fractionation. However, at the permissive temperature, 32% of [^{35}S]methionine-labeled p60^{src} is soluble in chicken cells transformed with *ts*LA29 (data not shown). *ts*LA29-infected

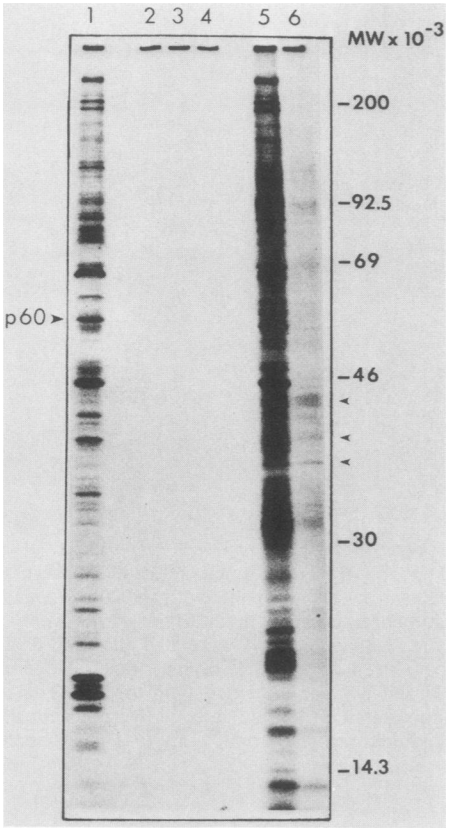

FIG. 2. Effect of cycloheximide on incorporation of [^{3}H]myristic acid and [^{3}H]palmitate into cellular proteins. SR-RSV-D-infected chicken cells were pretreated for the indicated times with 20 μg of cycloheximide per ml and then labeled in the continued presence of cycloheximide for 2 h with either [^{3}H]myristic acid or [^{3}H]palmitic acid. Cells were lysed directly in electrophoresis sample buffer, and samples containing 10^5 cells were analyzed by SDS-PAGE. Fluorographic exposure was for 14 days. p60^{src} is marked by an arrow, and the three proteins whose labeling with [^{3}H]palmitic acid was unaffected by cycloheximide are indicated. Lanes: 1, [^{3}H]myristic acid-labeled cells with no cycloheximide; 2, [^{3}H]myristic acid-labeled cells with cycloheximide and [^{3}H]myristic acid added simultaneously; 3, [^{3}H]myristic acid-labeled cells with 5 min of pretreatment with cycloheximide; 4, [^{3}H]myristic acid-labeled cells with 20 min of pretreatment with cycloheximide; 5, [^{3}H]palmitic acid-labeled cells with no cycloheximide; 6, [^{3}H]palmitic acid-labeled cells with 20 min of pretreatment with cycloheximide.

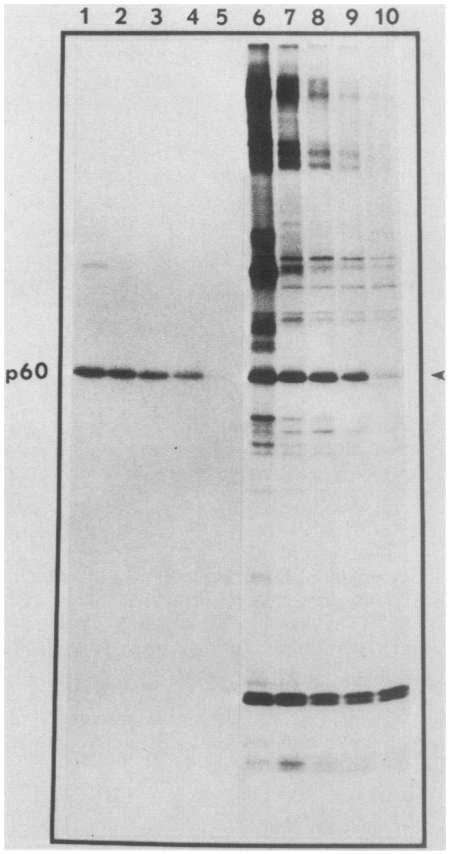

FIG. 3. Comparison of the turnover of p60^{src} labeled with [^{3}H]myristic acid or [^{35}S]methionine. SR-RSV-D-infected chicken cells were labeled for 2 h with [^{3}H]myristic acid or [^{35}S]methionine and then chased for 3 or 6 h in nonradioactive medium containing 0.18 mg of myristic acid (P-L Biochemicals, Inc.) per ml. Nonradioactive medium without the added myristic acid was then added to the plates used for the 10- and 22-h time points to prevent excessive accumulation of fatty acid. p60^{src} was immunoprecipitated with an excess of rabbit antitumor serum and analyzed by SDS-PAGE. The number of cells in both [^{3}H]myristic acid- and [^{35}S]methionine-labeled cultures doubled during the 24-h chase. The tracks containing the [^{3}H]myristic acid-labeled proteins were exposed for 5 days. The tracks containing the [^{35}S]methionine-labeled proteins were exposed for 1 day. Lanes: 1, cells labeled with [^{3}H]myristic acid for 2 h; 2, [^{3}H]myristic acid-labeled cells with a 3-h chase; 3, [^{3}H]myristic acid-labeled cells with a 6-h chase; 4, [^{3}H]myristic acid-labeled cells with a 10-h chase; 5, [^{3}H]myristic acid-labeled cells with a 22-h chase; 6, cells labeled with [^{35}S]methionine for 2 h; 7, [^{35}S]methionine-labeled cells with a 3-h chase; 8, [^{35}S]methionine-labeled cells with a 6-h chase; 9, [^{35}S]methionine-labeled cells with a 10-h chase; 10, [^{35}S]methionine-labeled cells with a 22-h chase.

chicken cells were labeled with [^{3}H]myristic acid and lysed in hypotonic buffer, and p60^{src} was isolated from particulate and soluble fractions generated by high-speed centrifugation. [^{3}H]myristate-labeled cell lysates were examined directly to ensure analysis of the entire population of p60^{src}. Thirty-six percent of the [^{3}H]myristic acid-containing p60^{src} molecules were found in the cytosol (Fig. 4). This demonstrates, in a more traditional manner, that myristic acid-modified p60^{src} can be cytosolic. The solubility of myristylated p60^{src} is not a unique property of the p60^{src} proteins encoded by temperature-sensitive mutant viruses. We also have detected small quantities of soluble, lipid-containing p60^{src} in cells transformed by wild-type SR-RSV-A and SR-RSV-D (data not shown).

More than 30 myristate-labeled cellular proteins can be resolved by SDS-PAGE. All of these myristylated proteins were present in the particulate fraction. Strikingly, at least half could also be detected in the cytosolic fraction, and almost a third of the proteins were in fact more abundant in the soluble fraction than in the particulate fraction. Myristylated proteins are therefore not restricted to cellular membranes.

Myristylation of mutant p60^{src} is not temperature sensitive. The p60^{src} encoded by the mutant virus *ts*NY68 is reported to contain less lipid at the nonpermissive than at the permissive temperature (13). However, this experiment was performed with [^{3}H]palmitic acid as a label, which we now know is incorporated into p60^{src} only after catabolism to [^{3}H]myristic acid (Buss and Sefton, in press). We have now

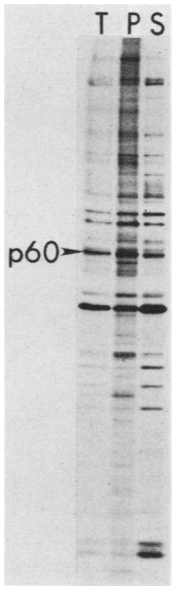

FIG. 4. [^{3}H]myristic acid-labeled proteins in soluble and particulate fractions. Chicken cells transformed by *ts*LA29 and grown at the permissive temperature (35°C) were labeled for 2 h with [^{3}H]myristic acid and homogenized and fractionated as described in the text. Fluorographic exposure was for 30 days. p60^{src} is indicated with an arrow. Lanes: T, unfractionated cell lysate; P, particulate fraction; S, soluble proteins.

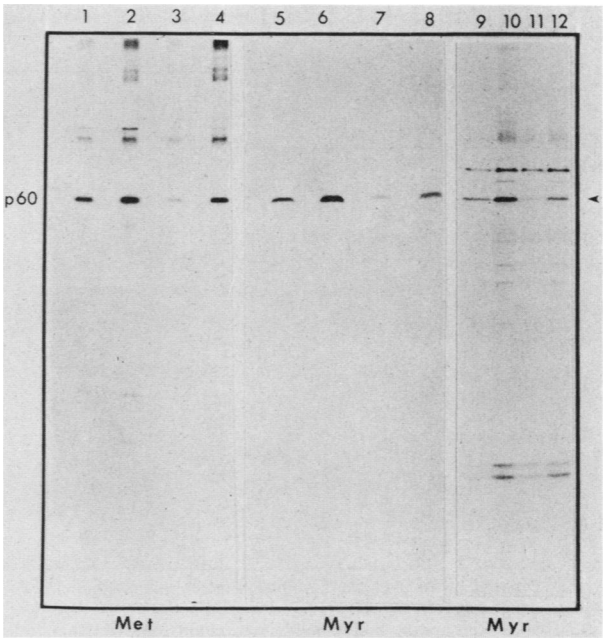

FIG. 5. [^{3}H]myristic acid incorporation into p60^{src} of *ts*NY68. Chicken cells infected with *ts*NY68 or SR-RSV-A were grown at 35 or 41°C as described in the text and labeled for 2 h with [^{3}H]myristic acid or [^{35}S]methionine. Immunoprecipitates were formed with antitumor serum and were resuspended in 50 μl of electrophoresis sample buffer. Proteins contained in 5 μl of [^{35}S]methionine-labeled immunoprecipitates or in 20 μl of [^{3}H]myristic acid-labeled immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. Exposure for the [^{35}S]methionine-labeled proteins was 1 day. Exposure of the immunoprecipitated [^{3}H]myristic acid-labeled proteins was for 1 day. The [^{3}H]myristic acid-labeled lysates were exposed for 5 days. Lanes: 1, [^{35}S]methionine-labeled cells, *ts*NY68, 35°C; 2, [^{35}S]methionine-labeled cells, *ts*NY68, 41°C; 3, [^{35}S]methionine-labeled cells, SR-RSV-A, 35°C; 4, [^{35}S]methionine-labeled cells, SR-RSV-A, 41°C; 5, [^{3}H]myristic acid-labeled cells, *ts*NY68, 35°C; 6, [^{3}H]myristic acid-labeled cells, *ts*NY68, 41°C; 7, [^{3}H]myristic acid-labeled cells, SR-RSV-A, 35°C; 8, [^{3}H]myristic acid-labeled cells, SR-RSV-A, 41°C; 9, total lysate from [^{3}H]myristic acid-labeled cells, *ts*NY68, 35°C; 10, total lysate from [^{3}H]myristic acid-labeled cells, *ts*NY68, 41°C; 11, total lysate from [^{3}H]myristic acid-labeled cells, SR-RSV-A, 35°C; 12, total lysate from [^{3}H]myristic acid-labeled cells, SR-RSV-A, 41°C.

examined whether the labeling of the mutant protein with [^{3}H]myristic acid was affected by temperature. Chicken cells infected with either *ts*NY68 or the parental wild-type virus SR-RSV-A were labeled for 2 h with [^{35}S]methionine or [^{3}H]myristic acid, and p60^{src} was isolated by immunoprecipitation with an excess of antitumor serum (Fig. 5). Because myristic acid appears to be added to p60^{src} immediately after the protein is synthesized (see above), comparison of the [^{35}S]methionine and [^{3}H]myristic acid incorporated into p60^{src} during this time will reveal accurately the extent to which p60^{src} becomes myristylated at the two temperatures. Because of the different growth rates of transformed and nontransformed cells and of cells grown at 35 and 41°C, the number of cells and therefore the amount of p60^{src} in the various cultures differed. The amount of [^{3}H]myristic acid-labeled p60^{src}, however, varied in parallel with the amount of [^{35}S]methionine-labeled p60^{src} (Table 1). The relative incorporation of [^{3}H]myristic acid into the mutant p60^{src} was not significantly different at 35 or 41°C and was, if anything, greater at 41 than at 35°C. The efficient incorporation of [^{3}H]myristic acid into the mutant p60^{src} at both 35 and 41°C was also apparent when [^{3}H]myristic acid-labeled lysates were analyzed directly (Fig. 5). The presence of [^{3}H]myristic acid in p60^{src} at the nonpermissive temperature was not due to leakiness of the mutant-infected cells. The *ts*NY68-infected cultures were completely transformed at 35°C but were morphologically normal at 41°C. Analysis of rates of 2-deoxyglucose uptake showed that the *ts*NY68-infected cells displayed the expected approximately fourfold greater rate of transport at 35 than at 41°C (data not shown).

We have also measured the fatty acylation of the p60^{src} protein of a second mutant virus, *ts*LA29, and its wild-type parent, PR-RSV-A, at both the permissive and restrictive temperatures. When labeled with [^{3}H]palmitic acid for 2 h, the amount of lipid in p60^{src} from either virus was slightly greater at 41 than at 35°C (Table 1).

It was possible that the 2-h labeling period failed to reveal steady-state differences in acylation of the mutant p60^{src} at 35 and 41°C. We have examined this question by labeling *ts*NY68-infected cells for 18 h with [^{3}H]palmitic acid. Here too we could detect no decrease in the lipid content of the p60^{src} protein of *ts*NY68 at the nonpermissive temperature (Table 1).

DISCUSSION

We have found here that myristic acid is added to p60^{src} in the cytosol before the deposition of the protein in membranes. The evidence for this is twofold. First, we could detect no lag between the release of the protein from soluble polysomes and the attachment of the myristyl moiety. Second, the population of newly synthesized p60^{src} associated with hsp90 and p50, the two cellular proteins which may mediate the movement of the protein to the plasma mem-

TABLE 1. Lack of effect of temperature on incorporation of lipid into temperature-sensitive forms of $p60^{src}$

Virus	Temp (°C)	Labeling time (h)	Incorporation of:					
			[^{3}H]myristic acid (cpm)[a]	[^{35}S]methionine (cpm)[a]	Myr/Met[b]	[^{3}H]palmitic acid (peak area)[c]	[^{35}S]methionine (peak area)[c]	Palm/Met[b]
*ts*NY68	35	2	294	272	1.08			
	41	2	596	452	1.32			
SR-RSV-A	35	2	125	81	1.54			
	41	2	271	292	0.94			
*ts*LA29	35	2				37	376	0.10
	41	2				65	484	0.13
PR-RSV-A	35	2				13	146	0.09
	41	2				29	224	0.13
*ts*NY68	35	18				16	77	0.21
	41	18				36	125	0.29
SR-RSV-A	35	18				37	36	1.04
	41	18				65	86	0.76

[a] $p60^{src}$-containing pieces of the 2,5-diphenyloxazole-impregnated polyacrylamide gel (Fig. 5) were counted directly in scintillation fluid. A background of 30 cpm has been subtracted.
[b] Myr/Met, [^{3}H]myristic acid/[^{35}S]methionine; Palm/Met, [^{3}H]palmitic acid/[^{35}S]methionine.
[c] Arbitrary units from densitometric scans of fluorograms similar to Fig. 5.

brane (4, 8), already contains myristic acid. Somewhat similar observations have also been made by Cross and colleagues (10).

Although we have not yet determined whether myristic acid is added during or immediately after the synthesis of the protein, we favor the idea that myristylation is a cotranslational process, as is the case for the acetylation of amino termini (11, 23, 28), and that the nascent polypeptide chain is the substrate of the myristyl transferase. It is quite possible, in fact, that nascent chains are the only substrates of N-terminal myristyl transferases. Inhibition of protein synthesis with cycloheximide halted the incorporation of myristic acid completely. This suggested that myristic acid groups do not undergo turnover. Pulse-chase analysis demonstrated directly that this is indeed the case for $p60^{src}$. We suggest that myristylation is a permanent form of protein modification which occurs as the nascent polypeptide emerges from the polysome.

We initially suggested that lipid was added to $p60^{src}$ to convert the protein from a soluble species to a membrane protein. Since it now appears that the addition of lipid to $p60^{src}$ precedes the interaction of the protein with the plasma membrane, this idea, at least in its simplest form, is clearly incomplete. What then is the function of the myristyl moiety in $p60^{src}$? There is still good reason to think that it may help anchor the protein to cellular membranes. Cross et al. (10) and Garber and colleagues (13) have shown that a number of variants of $p60^{src}$ which do not contain myristic acid bind poorly to membranes and also do not cause cellular transformation. This suggests that the presence of myristate may in fact be essential for membrane binding and that the presence of the myristyl moiety in $p60^{src}$ may be indispensible for transformation. Nevertheless, the presence of myristic acid in a protein appears not in itself to be sufficient to bind the protein to a lipid bilayer. Soluble $p60^{src}$ is abundant in cells infected with temperature-sensitive RSVs (4, 8, 12; our unpublished data) and, at both the permissive and nonpermissive temperature, contains myristic acid. A myristyl group then does not make $p60^{src}$ insoluble in the cytoplasm. The existence of soluble forms of $p60^{src}$ which contain myristic acid was not unanticipated. A number of other cellular proteins which contain myristic acid, including the catalytic subunit of the cyclic AMP-dependent protein kinase and calcineurin B, are found predominantly in the cytosol (Fig. 4; 6, 18). Myristylated proteins, including $p60^{src}$, thus are not invariably attached to the lipid bilayer. The myristyl group may have an additional function, quite unrelated to increasing the affinity of a protein for a lipid bilayer.

It is now clear that eucaryotic proteins undergo two very different forms of fatty acylation. Myristic acid is linked through an amide bond to the alpha-amino group of an amino-terminal glycine residue of $p60^{src}$ and a small number of other proteins (1, 6, 34). The myristylation of N-termini appears to occur very early in the life of a protein and may well take place before completion of the synthesis of the polypeptide. In the case of $p60^{src}$, the myristyl group appears to be metabolically stable and not to undergo turnover. The enzyme(s) which attaches fatty acids to amino-terminal glycines in general exhibits a strict specificity for myristic acid. Even when [^{3}H]palmitic acid is used to label $p60^{src}$, all of the inefficient labeling which is observed (39; Buss and Sefton, in press) reflects incorporation of [^{3}H]myristic acid arising from metabolism of the [^{3}H]palmitic acid (Buss and Sefton, in press). Furthermore, where chemical analysis has been possible, all of the fatty acid in the catalytic subunit, $p15^{gag}$, and calcineurin has been found to be myristic acid (1, 6, 15).

The modification of proteins with palmitic acid is a quite different process. Palmitic acid is not known to modify the amino termini of proteins. Rather it is often linked through alkali-labile bonds to internal amino acids within domains through which the protein is bound to the lipid bilayer. In the G glycoprotein of vesicular stomatitis virus, there is good reason to believe that palmitic acid is bound through a thioester bond to cysteine 488, a residue located in the short, cytoplasmic, carboxy-terminal tail of the protein (30). Palmitic acid appears to be attached to proteins only after their

release from the polysome. Several viral glycoproteins acquire palmitate in the Golgi apparatus after an obligatory lag of 20 min (33). Palmitylation is also clearly posttranslational for $p21^{ras}$ and the transferrin receptor (25, 39), because it is present only in the processed forms of these proteins. The enzyme(s) which palmitylates proteins also appears to utilize palmitic acid with some specificity. Chemical analysis of the G protein of vesicular stomatitis virus revealed that >80% of the fatty acid present was palmitate (32). Finally, palmitate is not necessarily a permanent protein modification. The palmityl moiety present in the transferrin receptor has been shown to undergo removal and replacement several times during the lifespan of the protein (25).

We have not been able to confirm that temperature-sensitive $p60^{src}$ proteins, which are largely cytosolic (12), contain a reduced amount of fatty acid at the nonpermissive temperature (13). We found that the acylation of the $p60^{src}$ proteins of both *ts*NY68 and *ts*LA29 viruses occurred equally well at 35 or 41°C. In addition, the abundance of fatty acid at steady state in the $p60^{src}$ protein of *ts*NY68 was identical at both the permissive and nonpermissive temperatures. It seems clear that the decreased ability of the mutant proteins to bind to cellular membranes at the restrictive temperature is not due to an absence of fatty acid and that the presence of myristic acid in $p60^{src}$ cannot be used as a means by which to distinguish soluble from membrane-bound forms of the enzyme.

There is recent evidence that $p60^{src}$ may phsophorylate phosphatidylinositol (40) as well as protein substrates. Since this phospholipid is confined to cellular membranes, binding to the plasma membrane could be important for the interaction of $p60^{src}$ with phosphatidylinositol. It is not unreasonable that the myristyl moiety in $p60^{src}$ plays a role in this interaction. Elucidation of the role of myristate in the several possible enzymatic activities of $p60^{src}$ should be facilitated greatly by site-directed mutations which prevent specifically the fatty acylation of the polypeptide.

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