The Absence of Myristic Acid Decreases Membrane Binding of p60^{src} but Does Not Affect Tyrosine Protein Kinase Activity

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We have constructed two point mutants of Rous sarcoma virus in which the amino-terminal glycine residue of the transforming protein, p60^{src}, was changed to an alanine or a glutamic acid residue. Both mutant proteins failed to become myristylated and, more importantly, no longer transformed cells. The lack of transformation could not be attributed to defects in the catalytic activity of the mutant p60^{src} proteins. *In vitro* phosphorylation of the peptide angiotensin or of the cellular substrate proteins enolase and p36 revealed no significant differences in the *K_m* or specific activity of the mutant and wild-type p60^{src} proteins. However, when cellular fractions were prepared, less than 12% of the nonmyristylated p60^{src} proteins was bound to membranes. In contrast, more than 82% of the wild-type protein was associated with membranes. Wild-type p60^{src} was phosphorylated by protein kinase C, a protein kinase which associates with membranes when activated. The mutant proteins were not. This finding supports the idea that within the intact cell the nonmyristylated p60^{src} proteins are cytoplasmic and suggests that this apparent solubility is not an artifact of the cell fractionation procedure. The myristyl groups of p60^{src} apparently encourages a tight association between protein and membranes and, by determining the cellular location of the enzyme, allows transformation to occur.

The transforming gene of Rous sarcoma virus encodes a 60,000-dalton (Da) tyrosine-specific protein kinase termed p60^{src} (9, 20, 24). A majority of the p60^{src} in transformed cells is bound to the inner surface of the plasma membrane (13, 22, 23). A small population of p60^{src} is present in the cytosol, and some is apparently bound to intracellular membranes (34). All forms of p60^{src} contain myristic acid, covalently bound through an amide linkage to the N-terminal glycine of the protein (5, 35). The initiating methionine of p60^{src} is removed, and the fatty acid is attached to the exposed penultimate glycine residue (35).

Myristylation is an unusual type of protein modification. One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis suggests that fewer than 50 proteins in avian and mammalian cells are myristylated to a detectable extent (5, 25, 27). In addition to p60^{v-src} and its cellular homolog p60^{c-src}, four other myristylated proteins have been identified: the catalytic subunit of the cyclic AMP-dependent protein kinase, the B subunit of the phosphatase calcineurin, NADH-cytochrome *b*₅ reductase, and several mammalian retroviral proteins which contain a p15^{gag} domain (1, 6, 19, 29, 36). The linkage of the myristyl group to the protein backbone is identical in each of these proteins: through an amide bond to the amino-terminal glycine residue.

These proteins have few characteristics in common from which we might deduce a function for myristic acid. Although several are involved in protein phosphorylation and dephosphorylation, this is not true of p15^{gag} or NADH cytochrome *b*₅ reductase. Nor are all myristylated enzymes found in the same subcellular location. The catalytic subunit of the cyclic AMP-dependent protein kinase, once released from its regulatory subunits, is freely soluble (6). Cytochrome *b*₅ reductase is microsomal (29), while the majority of p60^{src} is membrane associated (13, 22, 23).

We have constructed two mutants of p60^{src} in which the

codon for glycine 2, the amino acid to which the myristic acid is attached, is changed to one encoding either alanine (SD10/Ala) or glutamic acid (SD11/Glu) (21). Even the conservative substitution of an alanine for the glycine results in a protein which is not myristylated (21). Strikingly, both nonmyristylated mutant p60^{src} proteins are unable to induce morphological transformation of infected chicken cells. Hanafusa and colleagues have studied a series of mutant p60^{src} proteins with amino-terminal insertions and deletions (14, 16, 31). These mutants also are not myristylated and do not transform cells. These results suggest that the myristic acid at the amino terminus of p60^{src} plays an essential role in cellular transformation.

The mutants developed by Cross et al. (14) showed that the amino-terminal insertions and deletions of p60^{src} which prevent fatty acylation also interfere with membrane binding. We wished to know whether this was also true for p60^{src} proteins with more specific point mutations at the N terminus. We examined four possible ways in which the myristyl group might affect the function of p60^{src}. We asked whether the myristic acid (i) increased the stability of the protein; (ii) helped maintain the correct conformation of the active enzyme; (iii) assisted in the interactions between p60^{src} and substrate proteins; or (iv) stabilized the binding of p60^{src} to the plasma membrane. We found that the mutant, nonacylated p60^{src} proteins are fully functional as tyrosine kinases, recognize appropriate substrate proteins *in vitro*, and retain the kinetic characteristics of the wild-type enzyme. The only effect of the absence of the myristyl group which we detected was a loss of the binding of p60^{src} to cellular membranes.

MATERIALS AND METHODS

Viruses, cells, and antisera. The SD10/Ala and SD11/Glu viruses were generated by oligonucleotide-directed mutagenesis of the codon for glycine 2 of p60^{src} from the Prague strain of Rous sarcoma virus, subgroup C (21, 37). Chicken embryo cell cultures were prepared and infected as described

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previously (39). The extent of infection of nontransformed cultures was determined by measurement of viral reverse transcriptase activity in the growth medium (43) and by quantitative immunoprecipitation from infected cell lysates of the viral precursor protein, Pr76^{gag}, labeled with [³⁵S]methionine. p60^{src} was isolated by immunoprecipitation with an antiserum directed against the carboxy-terminal hexapeptide of p60^{src} (40). Antiserum to the heat shock protein hsp 90 was obtained from Milton Schlesinger, Washington University, St. Louis, Mo.

Biosynthetic labeling. Cells were labeled with [³⁵S]methionine for 15 h in Dulbecco-Vogt modified Eagle medium containing 25% or, for sedimentation analysis, 50% of the normal concentration of methionine and 4% calf serum. Labeling with ³²P_i was performed by incubation of cells for 15 h in the presence of 2 mCi of ³²P_i per ml in Dulbecco-Vogt modified Eagle medium containing 5% of the normal concentration of phosphate and 4% dialyzed calf serum. The phorbol ester tetradecanoyl phorbol acetate (TPA; 50 ng/ml) was added for 30 min at the end of the labeling period.

Immunoprecipitation and electrophoresis. Lysis and immunoprecipitation of p60^{src} from chicken cells in RIPA or Nonidet P-40 buffer (RIPA lacking SDS and deoxycholate) were done as described previously (38). Samples were analyzed by electrophoresis on 15% polyacrylamide gels (39) and detected by autoradiography or, for detection of [³⁵S]methionine-labeled proteins, by fluorography of a diphenyloxazole-impregnated gel. Pieces of the dried gel were excised and counted directly in 3a70B scintillation fluid (Research Products International Corp.) to quantify radioactive proteins.

Partial proteolytic mapping. One-dimensional peptide maps were prepared by digestion of p60^{src}-containing gel pieces with 25 or 250 ng of *Staphylococcus aureus* V8 protease during electrophoresis on a second 15% SDS-polyacrylamide gel (7).

Cell fractionation. All steps were carried out at 4°C. Cells were swollen in hypotonic buffer (14, 17) containing 10 mM KCl, 1 mM EDTA, 25 mM HEPES buffer (pH 7.4) and broken with 30 strokes in a tight-fitting Dounce homogenizer (4). The lysate was adjusted to 0.15 M NaCl (17), and soluble and particulate fractions were prepared directly from the cell lysate by centrifugation at 100,000 × g for 30 min at 4°C. p60^{src} from the separated fractions was isolated by immunoprecipitation in NP-40 buffer with 2 mM EDTA (NP-40-EDTA) to preserve kinase activity.

Sedimentation of p60^{src}. Cell lysates were prepared in NP-40-EDTA buffer, clarified, and sedimented through 5 to 20% glycerol gradients for 19 h at 40,000 × g in a Beckman SW50.1 rotor as described previously (4). Use of NP-40-EDTA buffer rather than RIPA allowed the anti-carboxy-terminal peptide serum to recognize and immunoprecipitate the complex of p60^{src}-p50-hsp 90 (40).

In vitro phosphorylation. p60^{src} immunoprecipitates were prepared in NP-40-EDTA buffer and washed with kinase buffer (25 mM sodium phosphate [pH 7.2], 10 mM MgCl₂). Samples for specific activity measurements were suspended in 10 μl of kinase buffer containing 12 μCi of [³²P]ATP (3,000 Ci/mmol; Amersham Corp.), 10 μM ATP, and 0.4 to 4 mM [Val⁵]-angiotensin II (Sigma Chemical Co.) and incubated at 30°C for 2, 4, or 6 min. Incorporation of radioactivity was linear with time throughout the reaction period. For determination of kinase activity in soluble or particulate fractions of cell lysates, assay mixtures containing 8 μCi of [³²P]ATP, 1 μM ATP, and 2 mM angiotensin were incubated for 5 min. Phosphorylated angiotensin was isolated by one-dimensional

electrophoresis at pH 3.5 on cellulose thin-layer plates (44) and detected by autoradiography. ³²P-containing angiotensin was scraped from the plate and counted by scintillation spectrometry.

For phosphorylation of the p36 protein and enolase, immunoprecipitates with equal angiotensin-phosphorylating activity were suspended in kinase buffer containing 15 μCi of [³²P]ATP, 20 μM ATP, and 9 μg of the complex of p36 and 10-kilodalton (10K) proteins (17a) or 15 μg of acid-denatured enolase (10). p36 was a generous gift from J. Glenney, Salk Institute, and was purified as described previously (17a). The concentration of p36 protein in the assay (2.4 μM) is approximately equal to the *K_m* for this substrate (17a), to allow subtle variations in the rate of phosphorylation to be detected. Reaction mixtures were incubated at 30°C for 10 min, during which time ³²P incorporation was linear. Phosphorylated p36 and enolase and autophosphorylated p60^{src} were separated by SDS-polyacrylamide gel electrophoresis.

Phosphoamino acid analysis and two-dimensional peptide mapping. The phosphoamino acid content of p60^{src}, isolated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, was determined as described previously (20). Tryptic phosphopeptides were generated from p60^{src} which had been purified on one-dimensional polyacrylamide gels, as described previously (2). Phosphopeptides were separated in two dimensions by electrophoresis at pH 8.9 and ascending chromatography (2).

RESULTS

Effects of the alanine 2 and glutamic acid 2 mutations on p60^{src} protein stability. One role of the myristic acid at the amino terminus of p60^{src} might be to protect the protein from premature degradation. We determined the amount of p60^{src} in cells infected with mutant viruses by labeling the cells with [³⁵S]methionine for 15 h and precipitating p60^{src} with an excess of antibody (Fig. 1). An abundant cellular protein, hsp 90 (28), was also isolated by immunoprecipitation to allow the differences in incorporation of [³⁵S]methionine between transformed and nontransformed cells to be normalized. When p60^{src}-containing immunoprecipitates were prepared from portions of cell lysates which contained equal amounts of labeled hsp 90, the amount of the mutant p60^{src} proteins averaged 42% (*N* = 5) of that of wild-type p60^{src}. Direct pulse-chase analysis of the rate of protein turnover was hampered by the presence of a contaminating protein which had a similar electrophoretic mobility to that of the mutant p60^{src}s on polyacrylamide gels. However, it was clear that cells infected with, but not transformed by, the mutant viruses contained a large amount of p60^{src} protein. This established that the absence of cellular transformation by the mutant viruses did not result from a lack of the transforming proteins.

Removal of the initiator methionine in mutant p60^{src} proteins. Because the initiator methionine of wild-type p60^{src} must be removed to expose the site of myristylation on the glycine residue, we examined whether either of the mutations affected this processing event. Partial proteolysis with *S. aureus* V8 protease cleaves p60^{src} into fragments with molecular masses of 36, 24, 20, and 18 kDa. The 36K, 20K, and 18K peptides contain the amino terminus of p60^{src} (8). Because the initiator methionine is removed, the 18K and 20K fragments of wild-type p60^{src} do not contain methionine and therefore are normally not detected when [³⁵S]methionine-labeled p60^{src} is analyzed (Fig. 1B) (35). The position of the 18K and 20K peptides was identified from the amino-

FIG. 1. (A) Amount of $p60^{src}$ in cells infected with mutant and wild-type virus. $p60^{src}$ was immunoprecipitated with anti-carboxy-terminal serum from equal numbers (10^6) of infected chicken cells labeled for 15 h with 100 μ Ci of [35 S]methionine per ml. An immunoprecipitate was also formed from uninfected cells to allow quantification of a contaminating protein which migrated near $p60^{src}$. hsp 90 was immunoprecipitated from duplicate aliquots of the cell lysates (data not shown). $p60^{src}$ samples derived from lysates containing equal amounts of hsp 90 were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by fluorographic exposure for 1 day. Radioactivity incorporated into gel pieces containing $p60^{src}$ was determined by scintillation counting. Lanes: 1, cells infected with SDWT virus (885 cpm); 2, SD10/Ala virus (415 cpm); 3, SD11/Glu virus (543 cpm); 4, uninfected cells (208 cpm). (B) Comparison of amino-terminal fragments of mutant and wild-type $p60^{src}$ by partial proteolysis. $p60^{src}$ was isolated by immunoprecipitation from cells labeled overnight with [35 S]methionine (SDWT, SD10) or [3 H]myristic acid (SDWT) or for 2 h with [35 S]methionine (SD11) and analyzed by SDS-polyacrylamide gel electrophoresis. The length of the labeling period had no effect on the results. Gel pieces containing $p60^{src}$ were excised, and the proteins were digested with 250 ng of *S. aureus* V8 protease during electrophoresis on a second polyacrylamide gel. Radioactive peptides were visualized by fluorographic exposure for 21 days. The 18K and 20K fragments which contain the amino terminal methionine are indicated. Lanes: 1, SD10/Ala $p60^{src}$, [35 S]methionine; 2, SDWT $p60^{src}$, [35 S]methionine; 3, SD11/Glu $p60^{src}$, [35 S]methionine; 4, SDWT $p60^{src}$, [3 H]myristic acid.

terminal fragments of [3 H]myristic acid-labeled wild-type $p60^{src}$. No methionine was detected in the 18K or 20K fragments of $p60^{src}$ from the SD10 mutant, although these fragments could be detected in 32 P-labeled SD10 $p60^{src}$ (data not shown). This implied that an alanine was the amino-terminal residue of the SD10 $p60^{src}$. For the SD11 mutant, in which a glutamic acid substitutes for glycine 2, proteolysis

TABLE 1. $p60^{src}$ kinase activity in cell fractions

$p60^{src}$	Angiotensin phosphorylation (cpm) ^a		Avg % particulate ^b
	Particulate	Soluble	
SDWT	22,111	5,512	82 ($n = 3$)
SD10	619	3,439	12 ($n = 3$)
SD11	481	8,493	6 ($n = 2$)

^a The data represent the average of duplicate samples from one experiment in which 5×10^6 or 1.5×10^6 cells from wild-type or mutant-infected cultures, respectively, were used.

^b The number of independent cell fractionation experiments (n) is indicated.

did yield 18K and 20K fragments which contained [35 S]methionine. It thus appeared that the presence of glutamic acid at position 2 either prevented or substantially inhibited the removal of the initiator methionine.

Lack of myristic acid decreases the binding of mutant $p60^{src}$ proteins to the plasma membrane. We compared the behavior of the nonmyristylated and wild-type $p60^{src}$ s during cell fractionation to determine whether the myristyl group played a role in binding the wild-type protein to cell membranes. Infected cells were swollen in hypotonic buffer containing 1 mM EDTA and homogenized, and the suspension was adjusted to 0.15 M NaCl. Proteins remaining attached to membrane fragments were separated from soluble proteins by centrifugation at $100,000 \times g$ for 30 min. $p60^{src}$ was immunoprecipitated from the separated fractions and quantified by measurement of the kinase activity in each fraction. As expected, the myristylated wild-type $p60^{src}$ protein was found predominantly in the particulate fraction, which contains both intracellular and plasma membranes (Table 1). In contrast, almost all of the activity of the nonacylated mutant proteins was recovered in the soluble fraction. The mutant $p60^{src}$ proteins clearly interacted less tightly with membranes than did myristylated $p60^{src}$.

Some $p60^{src}$ is normally present in the cytosol. Two cellular proteins, p50 and hsp 90, form a complex with $p60^{src}$ shortly after the protein is synthesized in the cytoplasm (3) and may act as a transport vehicle to shuttle $p60^{src}$ to the plasma membrane (3, 12). Since neither of the nonmyristylated mutant proteins was tightly bound to membranes, we wondered whether the apparently soluble nonmyristylated $p60^{src}$ s might accumulate in this complex. An SD10 cell lysate sedimented on a glycerol gradient (Fig. 2) contained $p60^{src}$ protein both as a monomer (fractions 12 through 16) and in association with p50 and hsp 90 (fractions 6 through 8). A total of 45% of the wild-type $p60^{src}$ and 30% of the SD10/Ala mutant $p60^{src}$ was found in the complex. The nonmyristylated SD10 mutant $p60^{src}$ protein thus formed a complex with p50 and hsp 90, but did not accumulate there.

The tyrosine kinase activity of nonmyristylated $p60^{src}$ s is equivalent to that of wild-type $p60^{src}$. Although both mutant $p60^{src}$ proteins produce extensive phosphorylation of tyrosine residues in cellular proteins, such phosphorylations do not lead to cellular transformation (21). We therefore examined whether the absence of the myristyl group caused a change in the enzymatic properties of the mutant proteins. First, the specific activities of the wild-type and mutant enzymes were compared in vitro by using [Val⁵]-angiotensin II as an exogenous substrate. Duplicate immunoprecipitates were formed from equal numbers of infected cells labeled overnight with [35 S]methionine. One sample was analyzed by SDS-polyacrylamide gel electrophoresis, and the other was assayed for enzyme activity. The maximal rates of

FIG. 2. Sedimentation of mutant and wild-type p60^{src}. Infected cells (2×10^6) were labeled for 15 h with 250 μ Ci of [35 S]methionine per ml, lysed in NP-40-EDTA buffer, and sedimented on glycerol gradients. p60^{src} in the individual fractions was isolated by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis. The fluorogram was exposed for 2 days. The direction of sedimentation was from right to left and is indicated by the horizontal arrow. p50 and hsp 90, which form the rapidly sedimenting complex with p60^{src}, are also indicated. (A) SD10/Ala; (B) SDWT.

angiotensin phosphorylation and the apparent affinity of each p60^{src} protein for the peptide substrate were determined (Table 2). In this experiment the mutant proteins displayed a slightly lower apparent K_m for the peptide (2 mM) than did wild-type p60^{src} (7 mM). In three additional experiments, the apparent K_m s of the mutant and wild-type enzymes were indistinguishable and varied from 3 to 10 mM. The interac-

TABLE 2. In vitro kinase activity of p60^{src}

p60 ^{src}	Angiotensin phosphorylation		Amt of [35 S]Met-p60 ^{src} (cpm) ^c	Sp act ^d
	Apparent K_m (mM) ^a	Kinase activity (cpm/min) ^b		
SD10	2.2	3,571	207	1.7
SD11	2.0	3,846	335	1.1
SDWT	6.7	6,250	677	0.9

^a Immunoprecipitated p60^{src} was used to phosphorylate Val⁵-angiotensin as described in Materials and Methods. K_m values were calculated from the x intercepts of lines fit by the least-squares method on double-reciprocal plots.

^b Because the amount of p60^{src} in the immunoprecipitates was unknown, the reciprocal of the y intercept represents a maximal rate of phosphorylation by these particular immunoprecipitates, rather than the V_{max} .

^c Aliquots of the samples used for phosphorylation were analyzed on SDS-polyacrylamide gels. [35 S]methionine-labeled p60^{src} was excised and counted.

^d The ratio of the rate of incorporation of 32 P into angiotensin (kinase activity) to the amount of [35 S]methionine-labeled p60^{src} in duplicate immunoprecipitates.

tion of the mutant enzymes with this peptide substrate thus was similar to that of the wild-type p60^{src}. We determined the specific activity of the three p60^{src} tyrosine kinases by calculating the ratio of kinase activity to the amount of [35 S]methionine-labeled p60^{src} present in the duplicate immunoprecipitates. The specific activities of the mutant and wild-type kinases were equivalent.

Although the [Val⁵]-angiotensin II peptide was useful for quantitative experiments, it is clearly not a normal cellular substrate for p60^{src}. We also tested the ability of the nonacylated mutant p60^{src}s to phosphorylate two proteins, p36 and enolase, which are substrates of p60^{src} in transformed cells (11, 15, 33). The p60^{src} proteins were first isolated by immunoprecipitation, and the kinase activity of each preparation was assayed with angiotensin as a substrate. The amounts of immunoprecipitates were adjusted to possess equal angiotensin-phosphorylating activity, and the immunoprecipitates were then assayed with p36 or acid-denatured enolase as substrate (Fig. 3). Both mutant and wild-type p60^{src} enzymes phosphorylated p36 and enolase at equivalent rates. We concluded that the absence of myristic acid had no effect on the intrinsic kinase activity of p60^{src} or its ability to interact with proteins which are authentic substrates in vivo.

Sites of phosphorylation of the mutant p60^{src} proteins. p60^{src} is itself phosphorylated by at least two protein kinases. The tyrosine residue at position 416 of p60^{src} is phosphorylated, probably through self phosphorylation (30, 41). Serine 17 is

phosphorylated by the cyclic AMP-dependent protein kinase (8). In addition, p60^{src} of the Prague strain of Rous sarcoma virus is phosphorylated at a second tyrosine, located either at position 205 or 208 (T. Patschinsky, T. Hunter, and B. M. Sefton, submitted for publication). The total incorporation of ³²P_i into SD10, SD11, and wild-type p60^{src} proteins was equivalent when normalized to the amount of [³⁵S]methionine-labeled protein (data not shown). The SD11/Glu mutant and wild-type proteins were each phosphorylated at three major sites (Fig. 4). The phosphorylation sites of p60^{src} from the SD10/Ala mutant were identical to those of the SD11/Glu protein (data not shown). Serine 17 is present in peptide alpha, tyrosine 416 is present in peptide beta, and the Prague-specific, phosphotyrosine-containing peptide is peptide delta (Patschinsky et al., submitted). The phosphoryla-

FIG. 3. In vitro phosphorylation of p36 and enolase proteins by mutant and wild-type p60^{src}. p60^{src} was isolated by immunoprecipitation in NP-40-EDTA buffer and assayed with angiotensin as a substrate, as described in Materials and Methods. Aliquots of the immunoprecipitates possessing equal angiotensin-phosphorylating activity were preincubated in 1 mM ATP for 20 min at room temperature in 10 mM sodium phosphate (pH 7.2)–5 mM MgCl₂ to reduce subsequent incorporation of isotope into p60^{src}. This preincubation had no differential effect on the kinase activity of wild-type or mutant p60^{src} (data not shown). The immunoprecipitates were then used to phosphorylate p36 (lanes 1 through 3) or enolase (lanes 4 through 6) for 10 min at 30°C. Phosphorylated p36 and enolase and the small residual amount of autophosphorylated p60^{src} (arrow) were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiographic exposure for 24 h. Lanes: 1, SD10; 2, SD11; 3, SDWT; 4, SD10; 5, SD11; 6, SDWT.

FIG. 4. Tryptic phosphopeptides of mutant and wild-type p60^{src} proteins. p60^{src} was isolated from infected cells labeled with 2 mCi of ³²P_i per ml for 15 h by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. In a separate experiment, TPA (50 ng/ml) was added to two cultures 30 min before the end of the labeling period. ³²P-labeled p60^{src} was eluted from the gels and digested with trypsin, and the peptides were separated by thin-layer electrophoresis at pH 8.9 and by ascending chromatography. Autoradiographic exposure was for 2 days. The arrow marks the point at which samples were applied. (A) SDWT p60^{src}; (B) SD11 p60^{src}; (C) SDWT p60^{src} from cells treated with TPA; (D) SD11 p60^{src} from cells treated with TPA.

tion of peptide alpha of the SD11/Glu mutant p60^{src} (Fig. 4B) indicated that the lack of the myristyl group at the amino terminus did not affect the phosphorylation of the nearby serine 17 residue.

Two new sites of phosphorylation in p60^{src} were recently discovered (18, 32). The Ca²⁺-phospholipid-stimulated protein kinase C modifies serine 12 and serine 48 of p60^{v-src}. The activity of protein kinase C can be stimulated by treating intact cells with the phorbol ester TPA. To determine whether the absence of the N-terminal myristic acid affected the phosphorylation of the mutant p60^{src}s by protein kinase C, we isolated p60^{src} from ³²P_i-labeled cells treated with TPA for 30 min. The phosphopeptides containing serine 12 (peptides 3 and 4) and serine 48 (peptide 5) could easily be seen in the tryptic digest of wild-type p60^{src} (Fig. 4C). No phosphorylation of the serine 12- or serine 48-containing peptides was detected in p60^{src} from TPA-treated cultures infected with SD10 (data not shown) or SD11 (Fig. 4D).

DISCUSSION

We have examined the stability, phosphorylation, enzymatic properties, and subcellular locations of two nonacyl-

ated point mutants of p60^{src}. The only marked effect of these mutations is to decrease the ability of the proteins to bind to membranes. Because the lack of myristic acid does not impair the enzymatic activity of these kinases, it would appear that the defect in transformation by these mutant viruses is largely due to the change in membrane binding of p60^{src}.

The amount of SD10/Ala or SD11/Glu p60^{src} present in mutant-infected cells is approximately 40% of the amount of SDWT p60^{src}. The somewhat lower abundance of both mutant proteins suggests that the myristyl moiety itself, or the association of p60^{src} with cell membranes that it induces, may slightly increase the lifetime of the polypeptide. The still substantial amounts of the nonacylated proteins in infected cells suggest that the failure of the mutant viruses to transform cells is unlikely to arise from an insufficient amount of p60^{src}. Indeed, the amounts of the mutant proteins in infected cells are fully sufficient to induce high levels of tyrosine phosphorylation of a number of cellular substrates of p60^{src} (M. P. Kamps, J. E. Buss, and B. M. Sefton, Cell, in press).

The most dramatic effect of the absence of myristic acid is the loss of association of p60^{src} with cell membranes. Without a myristyl group, the mutant proteins do not bind stably to cellular membranes, and are apparently cytosolic. This implies that the myristyl group plays an important role in binding wild-type p60^{src} to membranes and suggests that the interaction of p60^{src} with membranes is at least in part hydrophobic.

The phosphorylation of the mutant proteins on serine 17 by the cyclic AMP-dependent protein kinase and the autophosphorylation of tyrosine 416 are unchanged from those of wild-type p60^{src}. Serine residues 12 and 48 of wild-type p60^{src} are phosphorylated by protein kinase C (18). In contrast, neither serine 12 nor serine 48 becomes phosphorylated in either mutant protein when infected cells are treated with TPA. This difference in phosphorylation most probably reflects the different locations of the wild-type and nonacylated p60^{src}s in infected cells. The activated form of protein kinase C is associated with cell membranes (26). We suspect that the nonmyristylated p60^{src}s would be suitable substrates of protein kinase C were they associated with membranes. First, the mutant proteins can be phosphorylated by protein kinase C after immunoprecipitation (18). Second, serine 17 is phosphorylated normally, indicating that the N terminus of the mutant proteins is still available for interaction with (soluble) protein kinases. The behavior of proteins during cell fractionation is subject to many potential artifacts. However, the failure of the mutant proteins to be phosphorylated by a membrane-bound kinase within the intact cell is a further indication that the lack of myristic acid actually changes the physical location of the mutant p60^{src}s, rather than altering the behavior of the protein only during cell fractionation.

The nonacylated SD10 p60^{src} protein is found in a complex with hsp 90 and p50 to a similar extent as is wild-type p60^{src}. The lack of myristic acid does not, therefore, noticeably affect the interaction of p60^{src} and these two (soluble) proteins. Because 30% of the SD10/Ala protein is found in the complex, the remaining 70% must be present in the cytosol as a monomer. The mutant p60^{src} apparently retains a conformation which, like the wild-type protein, permits both the formation of the complex and the subsequent dissociation of its components (3, 12).

The SD10/Ala and SD11/Glu mutants illustrate two methods by which myristylation can be prevented: interference

with the first step of the reaction, removal of the initiator methionine; or direct failure of the amino acid which replaces the glycine to be a substrate for the myristyl transferase. The mutation in SD11/Glu prevents both myristylation and removal of the initiating methionine. The SD10/Ala mutation does not inhibit removal of the methionine. The aminopeptidase responsible for removal of initiator methionines of proteins in chicken cells thus appears to have some of the same properties as the methionine aminopeptidase of yeasts (42), that is, it will remove the methionine when the adjoining amino acid is an alanine, but not when the second residue is a glutamic acid. Four of the previously described nonmyristylated mutant p60^{src} proteins retain the initiator methionine (16) and thus resemble the SD11/Glu p60^{src}. The SD10/Ala mutant is the first example of a p60^{src} protein in which the glycine has been altered and the methionine is still removed. That even an alanine residue fails to become myristylated indicates that the myristyl transferase is uncommonly strict in its selection of substrates and perhaps can accommodate or recognize only glycine residues.

The mutant proteins are as active catalytically as the wild-type p60^{src}. In vitro, the mutant p60^{src}s will phosphorylate the peptide angiotensin and the cellular substrate proteins p36 and enolase at rates equivalent to that of the wild-type p60^{src}. Because there appear to be no defects in the intrinsic enzyme activity of the mutant p60^{src}s, the changes in substrate phosphorylation which we have observed in intact mutant-infected cells (Kamps et al., in press) most probably result only from the change in location of the nonacylated p60^{src}s.

These mutant viruses are useful for the study of how changes in substrate phosphorylation relate to the inability of the nonmyristylated p60^{src}s to cause transformation. It is clear that the lack of transformation does not result from a loss or change in the kinase activity of the nonmyristylated p60^{src} proteins. However, the almost total loss of biological activity of p60^{src} as a result of the absence of the myristyl group and membrane association of the protein indicates that the cellular location of p60^{src} can profoundly affect its transforming potential. It is also clear that the elevation of cellular phosphotyrosine produced by the tyrosine kinase activity of the mutant p60^{src} is, by itself, inadequate for cellular transformation.

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