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Mutation of NH_2-terminal glycine of $p60^{src}$ prevents both myristoylation and morphological transformation

(myristic acid/oligonucleotide-directed mutagenesis/tyrosine protein kinase/Rous sarcoma virus)

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ABSTRACT **$p60^{src}$, the transforming protein kinase of Rous sarcoma virus, contains the 14-carbon saturated fatty acid, myristic acid, linked through an amide bond to the α-amino group of its NH_2-terminal glycine residue. Myristic acid is known to be attached to four other eukaryotic proteins. In each case the fatty acid is also linked through an amide bond to an NH_2-terminal glycine. We have used oligonucleotide-directed mutagenesis to examine the amino acid specificity of the enzyme that myristoylates the NH_2 terminus of these proteins. Replacement of the NH_2-terminal glycine in $p60^{src}$ with either alanine or glutamic acid prevented myristoylation completely. This indicates that the myristoylating enzyme may have an absolute specificity for glycine. Strikingly, neither nonmyristoylated mutant src protein induced morphological transformation of infected cells, even though wild-type levels of phosphorylation of cellular proteins on tyrosine were observed in these cells. Since conversion of the NH_2-terminal residue from glycine to alanine should have little effect on the conformation of $p60^{src}$, the inability of this mutant $p60^{src}$ protein to induce morphological transformation suggests that the myristoyl moiety is essential for the transforming activity of the protein.**

Cellular transformation by Rous sarcoma virus results from the expression of a single viral protein designated $p60^{src}$ (1). $p60^{src}$ functions as a tyrosine-specific protein kinase (2, 3) *in vivo* and is also reported to phosphorylate phosphatidylinositol *in vitro* (4). Tyrosine phosphorylation may be crucial in the control of cellular proliferation. The mitogens epidermal growth factor (5), platelet-derived growth factor (6), and insulin-like growth factor (7) all stimulate tyrosine protein kinase activity when they bind to their cell-surface receptors. Immunofluorescence (8), immunoelectron microscopy (9), and cell fractionation (10–12) all suggest that a significant fraction of the $p60^{src}$ in transformed cells is associated with the cytoplasmic face of the plasma membrane. $p60^{src}$ may therefore deliver an unregulated mitogenic signal through the continuous phosphorylation of one or more proteins involved in the normal regulation of proliferation.

$p60^{src}$ is bound firmly to cellular membranes yet contains no large cluster of hydrophobic amino acids similar to those which are responsible for anchoring membrane-bound proteins such as the HLA and H-2 glycoproteins (human and murine major histocompatibility proteins) to a lipid bilayer. $p60^{src}$ does, however, contain covalently bound myristic acid, a rare 14-carbon saturated fatty acid (13). This myristic acid moiety is attached by an amide linkage to the α-amino group of the NH_2-terminal glycine residue of $p60^{src}$ (14). Consequently, an attractive hypothesis is that the hydrophobic myristoyl group plays a role in binding $p60^{src}$ to membranes.

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Myristoylation is an uncommon form of protein modification. Nevertheless, the amino acid to which myristic acid is attached has been identified unambiguously in four additional proteins. The catalytic subunit of the cyclic AMP-dependent protein kinase (15), calcineurin B (16), NADH-cytochrome b_5 reductase (17), and the $p15^{gag}$ structural protein of mammalian retroviruses (18) each contain a myristic acid moiety linked through an amide bond to the α-amino group of an NH_2-terminal glycine. Since every example of protein myristoylation involves the acylation of an NH_2-terminal glycine, it is possible that only glycine residues can be myristoylated. On the other hand, amino acids adjacent to the site of myristoylation could provide the determinants that the cellular myristoyltransferase recognizes, resulting in the acylation of any NH_2-terminal amino acid (with the exception of proline) at its free α-amino group. To examine whether an NH_2-terminal glycine is essential for protein myristoylation, we have used oligonucleotide-directed mutagenesis to convert the NH_2-terminal glycine in $p60^{src}$ to alanine and glutamic acid.

MATERIALS AND METHODS

Site-Specific Mutagenesis. Oligonucleotide-directed mutagenesis of the codon for glycine-2 of $p60^{src}$ was performed with viral DNA from the Prague strain of Rous sarcoma virus subgroup C which had been cloned originally in pBR322 from unintegrated circular viral DNA (19). This clone is designated pATV-8. To accomplish the mutagenesis, a 2.7-kilobase-pair *Sst* I fragment encompassing the *src* gene and 3′ long terminal repeat was removed from pATV8 and inserted into the polylinker of M13mp10. Site-directed mutagenesis (20) was performed with single-stranded M13 virion DNA using two oligonucleotides, both 18 residues long, which encompassed the codon for glycine-2.

Isolation of Mutant Viruses. The mutagenized 2.7-kilobase-pair *Sst* I fragment, excised from the replicative form of M13, was used to replace the wild-type fragment in the original plasmid pATV8. The complete viral DNA insert was then excised by partial digestion with restriction endonuclease *Hin*dIII. Because the viral genes are permuted in pATV8, the excised DNA was concatemerized prior to transfection (21) into chicken embryo fibroblasts. Virus stocks were harvested from transfected cultures and used to infect chicken cells.

Biosynthetic Labeling. Methionine labeling was achieved by growing cells for 2 hr in the presence of [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq) in 1 ml of Dulbecco–Vogt modified Eagle's medium (DMEM) containing 20% the normal concentration of methionine and 4% calf serum. Labeling with myristic acid was accomplished by incubating cells for 2 hr in the presence of 1 mCi of [^{3}H]myristic acid in 1 ml of DMEM as described (13). Cells were labeled with [^{32}P]P_i by incubation for 15 hr in the presence of 1.0 mCi of [^{32}P]P_i in 1 ml of DMEM containing 5% the normal concentration of

phosphate and 4% calf serum (previously dialyzed against phosphate-buffered saline).

Phospho Amino Acid Analysis. Quantification of phosphorylated amino acids from cellular protein extracts was performed as described (3).

RESULTS AND DISCUSSION

Mutagenesis of the NH_2 Terminus of $p60^{src}$. The NH_2 terminus of $p60^{src}$ has the structure myristoyl-Gly-Ser- (14). Using site-directed mutagenesis, we have replaced the codon for the NH_2-terminal glycine with one for either alanine or glutamic acid and have designated viruses encoding these mutant src proteins SD10 and SD11, respectively. The sequence of the *src* gene of the parental virus (SDWT) begins ATG-GGG- (Met-Gly-) (Fig. 1). The sequence of the mutant *src* gene in SD10 begins ATG-GCA- (Met-Ala-) and that in SD11 ATG-GAA- (Met-Glu-). We have determined the sequence of the DNA that encodes the first 264 amino acids of each of the mutant $p60^{src}$ proteins and found no changes other than the mutations at codon 2. Stocks of infectious virus were obtained from the mutagenized DNA by transfection of chicken cells.

Neither Mutant Protein Is Myristoylated. Both mutant $p60^{src}$ proteins were examined for the presence of covalently bound myristic acid by biosynthetic labeling of infected chicken cells with [^{3}H]myristic acid and immunoprecipitation. [^{35}S]Methionine-labeled mutant $p60^{src}$ proteins were easily identified. Both had slightly lower apparent molecular weights than did the wild-type protein. Neither was labeled detectably with [^{3}H]myristic acid (Fig. 2). Clearly, each mutation produced a $p60^{src}$ that was not a substrate for the myristoyltransferase.

Myristoylation of wild-type $p60^{src}$ occurs at glycine-2 after the initiating methionine residue is removed. To determine whether the initiating methionine residue was also removed from the mutant $p60^{src}$ proteins, we subjected them to partial proteolysis with *Staphylococcus aureus* V8 protease. *S. aureus* V8 protease produces NH_2-terminal 18- and 20-kDa fragments (23) that contain no methionine residues in wild-type $p60^{src}$ (24). Partial proteolysis of $p60^{src}$ encoded by SD10 virus produced no [^{35}S]methionine-labeled 18- or 20-kDa fragments. The initiating methionine therefore is apparently removed from the mutant $p60^{src}$ protein and subsequent myristoylation of the exposed alanine residue does not occur. In contrast, digestion of the $p60^{src}$ protein of SD11 virus yielded [^{35}S]methionine-labeled 18- and 20-kDa fragments. It is unclear whether cleavage of the initiating methionine residue is merely inefficient in this case or the sequence NH_2-Met-Glu- is not an appropriate substrate for the cellular aminopeptidase that cleaves most initiating methionine residues. The NH_2-terminal methionine of $pr76^{gag}$, the precursor to the internal structural proteins of avian retroviruses, is also followed by a glutamic acid residue and is not removed from the protein (25).

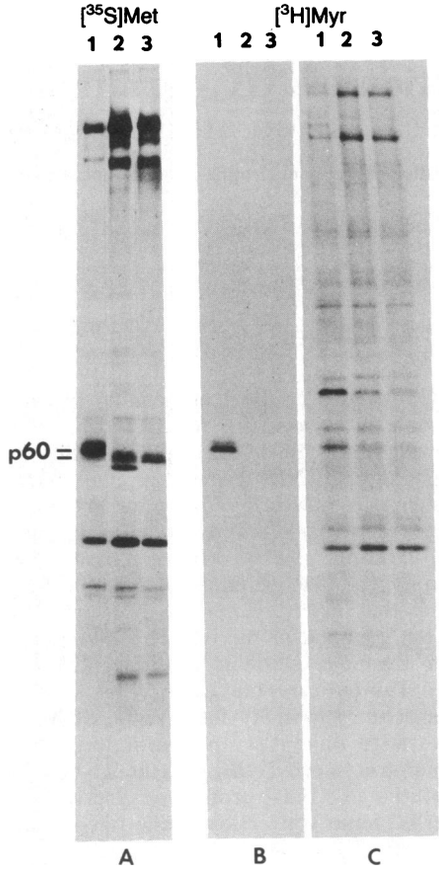

FIG. 2. Neither mutant $p60^{src}$ protein contains myristic acid. $p60^{src}$ was immunoprecipitated with an excess of antiserum reactive against the COOH terminus of the protein (22) and analyzed by $NaDodSO_4$/PAGE and fluorography. Cells were infected with wild-type SDWT (lanes 1) or with mutant SD10 (lanes 2) or SD11 (lanes 3) and metabolically labeled with [^{35}S]methionine (*A*) or [^{3}H]myristic acid (*B* and *C*). (*A* and *B*) Immunoprecipitates. (*C*) Total cellular proteins.

$p60^{src}$ Lacking Myristic Acid Does Not Induce Morphological Transformation. Chicken cells infected with either of the mutant viruses underwent no noticeable morphological change (Fig. 3). The inability of these two mutant viruses to transform cells could have resulted from a second, unintended mutation in the *src* gene. To examine this possibility, a fragment of SD10 and SD11 DNA, which had been found by DNA sequence analysis to contain only a mutation in codon 2, was replaced with the homologous fragment of wild-type viral DNA. These reconstructed viral DNAs were then

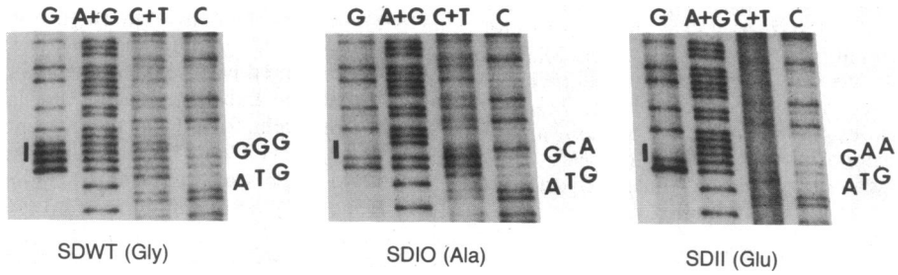

FIG. 1. Determination of the sequence of the mutant and wild-type *src* genes. Vertical bar at the left of each autoradiogram indicates the mutagenized codon. Also indicated is the ATG codon at which translation is initiated. Specific Maxam–Gilbert base-modification reactions are indicated at the top of each lane. The identity of each viral DNA and the amino acid at position 2 are indicated at the bottom.

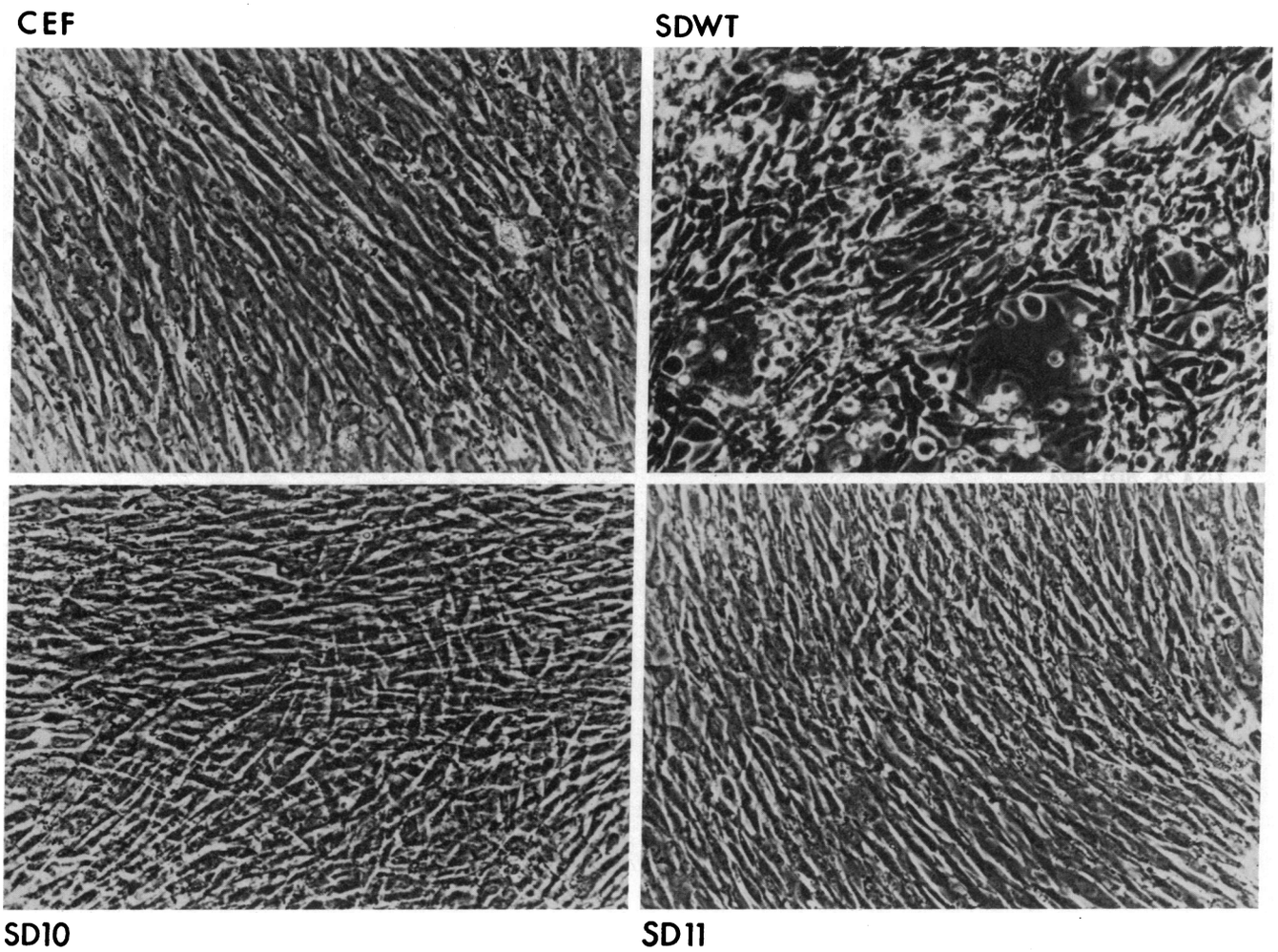

FIG. 3. Mutants SD10 and SD11 do not induce morphological transformation. Chicken embryo fibroblasts were infected with either SDWT, SD10, or SD11 and photographed when fully infected, as determined by the amount of viral reverse transcriptase in the medium. The panel labeled CEF shows uninfected chicken embryo fibroblasts. (×158.)

assayed for transforming activity by transfection into NIH 3T3 cells. The wild-type fragment restored transforming activity to both mutant viral DNAs. This proved that the transformation-defective phenotype of the mutants resulted specifically from the mutation in the codon for glycine-2.

$p60^{src}$ Lacking Myristic Acid Has Undiminished Protein Kinase Activity. To assay the activity of $p60^{src}$ as a tyrosine protein kinase in these infected cells, we measured the amount of phosphotyrosine present in total cellular protein. Phosphotyrosine typically increases 5- to 10-fold in cells transformed by Rous sarcoma virus (3). Cells infected with the mutant viruses contained as much, or more, phosphotyrosine as those infected with SDWT (Table 1). The transformation-defective phenotype does not, therefore, result from a defect in intrinsic tyrosine protein kinase activity of the mutant enzymes. Apparently, interaction of $p60^{src}$ with one or more crucial substrates cannot occur, but the phosphorylation of the majority of substrates continues unabated.

Table 1. Abundance of phosphotyrosine in mutant-infected cells

Virus	Phosphotyrosine, % of total acid-stable phospho amino acids
None	0.042
SD10	0.284
SD11	0.275
SDWT	0.187

CONCLUSIONS

These results demonstrate that the presence of an NH_2-terminal glycine is essential for the myristoylation of $p60^{src}$. That an alanine residue apparently cannot substitute for a glycine residue indicates that the enzyme which carries out this reaction has pronounced specificity both for acceptor amino acid as well as for donor fatty acid, which is almost exclusively myristic acid (13). We predict that NH_2-terminal glycine will also be found to be essential to the myristoylation of other proteins that contain this moiety.

More importantly, these results demonstrate that $p60^{src}$ requires covalently bound myristic acid to induce morphological transformation. Cells infected with these mutant viruses also do not grow when suspended in agar (unpublished observations). Myristoylation of $p60^{src}$ appears therefore to be necessary for several aspects of cellular transformation. Cross *et al.* (26) have reached a similar conclusion from studies of mutants of Rous sarcoma virus that contain small deletions and insertions in the NH_2-terminal 15 amino acids of $p60^{src}$. An absence of myristic acid apparently has no effect on the intrinsic tyrosine protein kinase activity of $p60^{src}$. Rather, it may prevent interaction of the protein with particular substrates whose phosphorylation is critical for transformation. This most probably results from a weakened association of the nonacylated $p60^{src}$ proteins with the plasma membrane. In contrast to the particulate wild-type protein,

both mutant proteins behave as cytosolic polypeptides during traditional cell fractionation (unpublished data).

Since extensive phosphorylation of tyrosine residues in polypeptides occurs in cells containing these nonacylated $p60^{src}$ proteins without induction of frank transformation, a thorough analysis of the specific proteins that become phosphorylated should allow us to distinguish between proteins whose phosphorylation is required for transformation by $p60^{src}$ and others that are simply adventitious substrates.

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