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The Six Amino-Terminal Amino Acids of p60^{src} Are Sufficient To Cause Myristylation of p21$^{v\text{-}ras}$

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We have used oligonucleotide-directed mutagenesis to replace the N-terminal amino acids of p21$^{v\text{-}ras}$ with residues which mimic the amino terminus of p60$^{v\text{-}src}$. p21$^{v\text{-}ras}$ protein possessing only the first five amino acids of p60^{src} was not myristylated, while substitution of residue 6 (serine) produced a protein p21(GSSKS) which incorporated [^{3}H]myristic acid that was stable to hydroxylamine, sensitive to inhibitors of protein synthesis, and found in both the normally nonacylated precursor and mature forms of p21(GSSKS). This defines the minimum framework of the p60$^{v\text{-}src}$ myristylation signal (glycine 2 and serine 6) and identifies serine 6 as a crucial part of that signal for myristylation of a protein in vivo.

Examination of the amino-terminal sequences of more than a dozen known myristylated proteins fails to reveal any consensus which might provide a reliable signal for an acyltransferase (24). An amino-terminal glycine is the only conserved residue and is the site of attachment of the myristyl group via an amide linkage (20). The only other discernible motif is the presence of a serine or threonine four residues from the myristylated glycine, which is found in 37 of 39 myristyl proteins (6, 21, 24). The signal for myristylation does, however, exist in the primary sequence of amino acids near the amino terminus of the protein, as addition of the first 15 amino acids of p60$^{v\text{-}src}$ to heterologous proteins produces a protein that is myristylated (18). The myristylation signal has also been studied in vitro by using synthetic peptides which mimic the amino termini of known myristylated proteins. This work has disclosed that the presence of a serine in position 5 (amino acid 6 of the protein if the initiating methionine is counted) greatly increases the binding of the peptide to the *N*-myristyl transferase (28–30). Our study was undertaken to determine if these properties of peptide myristylation hold true during the cotranslational addition (2, 31) of myristate in vivo.

The minimum signal for myristylation is contained in the first six amino acids of p60$^{v\text{-}src}$. Harvey sarcoma virus DNA (9) was modified by oligonucleotide-directed mutagenesis to replace p21$^{v\text{-}H\text{-}ras}$ codons 2, 3, and 4 with nucleotides encoding amino acids from the corresponding positions in p60$^{v\text{-}src}$ (Table 1). Position 5 in both proteins was a lysine residue. This DNA, designated p*ras*(GSSKL) was used to produce a second gene [p*ras*(GSSKS)] in which position 6 (leucine) was replaced by a serine. The coding sequence of each DNA was confirmed by dideoxy sequencing (19). Plasmid DNA was transfected into NIH 3T3 cells by the calcium phosphate precipitation technique (7), and morphologically transformed cells were isolated. p*ras*(GSSKS) DNA generated foci with a lower efficiency than wild-type or p*ras*(GSSKL) DNAs (C. J. Der and J. E. Buss, manuscript in preparation). The six introduced amino acids, therefore, did not abolish transformation by viral Harvey *ras* protein.

Myristylation of the mutant proteins was examined by immunoprecipitation of the proteins from cells labeled with [^{3}H]myristic acid (Dupont NEN Research Products) (23, 25). To distinguish the introduced proteins from the endogenous p21$^{c\text{-}K\text{-}ras}$, a monoclonal antibody (146-3E4; NCI Repository, Microbiological Associates, Bethesda, Md.) directed specifically against the carboxy-terminal residues 157 to 181 of p21$^{v\text{-}H\text{-}ras}$ was used. No ^{3}H-labeled p21(GSSKL) could be detected (Fig. 1, lane 3), even after very long exposures of the fluorogram (data not shown). In contrast, p21(GSSKS) did incorporate label, into both the mature and the precursor forms of the protein (Fig. 1, lane 5).

Because p21$^{v\text{-}H\text{-}ras}$ can be fatty acid acylated by a palmitic acid attached via a thioester to a cysteine residue near its carboxyl terminus (5) (a site retained in the mutant proteins), it was important to determine if the observed labeling of p21(GSSKS) with [^{3}H]myristic acid reflected the conversion of the fatty acid to [^{3}H]palmitic acid and subsequent attachment at the C-terminal site. The presence of label in the precursor form of the p21(GSSKS) protein, a form which does not contain palmitate (25), suggested that [^{3}H]myristic acid was the incorporated fatty acid. The palmitic acid of p21$^{v\text{-}H\text{-}ras}$ is attached some time after the protein is synthesized (16, 25). Inhibition of protein synthesis was used as a way to distinguish between cotranslational incorporation of myristic acid at the amino terminus (2) or posttranslational palmitate attachment to carboxy-terminal sites. Incorporation of label into p21(GSSKS) was abolished when [^{3}H]myristic acid-labeled cells were treated with emetine (Fig. 1, lane 6). This implied that [^{3}H]myristic acid was attached while the protein was being synthesized. Finally, to determine if the [^{3}H]myristic acid was attached via an amide or thioester bond, a duplicate sample of [^{3}H]myristic acid-labeled p21(GSSKS) protein was treated with hydroxylamine (3) (Fig. 1, lane 7). Under these conditions, >95% of the thioester-linked palmitate in p21$^{c\text{-}ras}$ or the transferrin receptor is removed (3). The complete stability of the incorporated [^{3}H]myristic acid suggested that the fatty acid was linked via an amide bond. Thus, the label incorporated into p21(GSSKS) in [^{3}H]myristic acid-labeled cells had all the expected characteristics of an N-terminal myristyl group.

This defines the minimum number of p60$^{v\text{-}src}$ residues which can act as a myristylation signal—Met-Gly-Ser-Ser-Lys-Ser. Because the initiating methionine is removed prior to attachment of the myristyl group (10), the signal appears to be five amino acids long. p21(GSSKS) also demonstrates directly that serine 6 is crucial for myristylation of an intact protein, as the otherwise identical p21(GSSKL), which has a

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TABLE 1. Comparison of nucleotide and predicted amino acid sequences of mutant genes

Gene	Nucleotide and predicted amino acid sequence[a]	Protein
v-H-*ras*	ATG ACA GAA TAC AAG CTT GTG GTG GTG Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val- 1 2 3 4 5 6 7 8 9	$p21^{v\text{-}H\text{-}ras}$
ras(GSSKL)	ATG GGC TCC TCC AAG CTT GTG GTG GTG Met-**Gly-Ser-Ser**-Lys-Leu-Val-Val-Val-	p21(GSSKL)
ras(GSSKS)	ATG GGC TCC TCC AAG TCC GTG GTG GTG Met-**Gly-Ser-Ser**-Lys-**Ser**-Val-Val-Val-	p21(GSSK*S*)
v-*src*	ATG GGG AGC AGC AAG AGC AAG CCT AAG Met-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys *	$p60^{v\text{-}src}$

[a] Nucleotide (upper lines) and predicted amino acid sequences (lower lines) of viral Harvey *ras*, the mutated *ras*, and *src* genes. Nucleotide differences introduced sequentially into the two mutated genes are underlined, and amino acids which differ from $p21^{v\text{-}ras}$ are in bold type. The glycine of $p60^{v\text{-}src}$ to which the myristyl group is attached after removal of the initiating methionine is marked with an asterisk.

leucine at this position, was not myristylated detectably [less than one-tenth as well as p21(GSSKS)].

Myristylation does not prevent maturation or cause premature membrane binding of p21(GSSKS). Pulse-chase labeling and fractionation in 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, 1% Trasylol, 0.1 mM phenylmethylsulfonyl fluoride, and 20 μg each of leupeptin, soy bean trypsin inhibitor, tolylsulfonyl lysyl chloromethyl ketone, and tolylsulfonyl phenylalanyl chloromethyl ketone per ml (1) showed that the precursor and one intermediate form of both p21(GSSKS) and $p21^{v\text{-}H\text{-}ras}$ were cytosolic (Fig. 2, lanes 1 and 5). None of the p21(GSSKS) precursor could be detected in the P100 (membrane-containing) fraction. Two rapidly migrating (fully mature) forms of p21(GSSKS) were found in the particulate fraction (Fig. 2, lanes 2 and 4). A difference in the electrophoretic mobilities of myristylated and nonmyristylated versions of $p60^{v\text{-}src}$ has been reported previously (13). If the presence or absence of a myristyl group explains the differences in mobility, then the nearly equal amounts of the two forms are consistent with the 38% value calculated below for

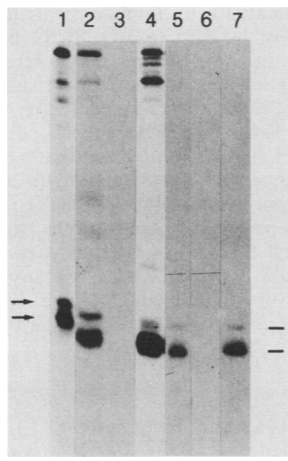

FIG. 1. p21(GSSKS) but not p21(GSSKL) contains myristic acid. Approximately 10^6 cells were labeled for 3.5 h with ^{35}S-TransLabel (100 μCi/ml; ICN Pharmaceuticals Inc.) or [^3H]myristic acid (1 mCi/ml). Two cultures were pretreated for 30 min and labeled in the continued presence of 20 μg of emetine per ml. $p21^{ras}$ proteins were isolated by immunoprecipitation with monoclonal antibody 146-3E4 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A duplicate gel containing [^3H]myristic acid-labeled p21(GSSKS) protein was treated with hydroxylamine. Proteins were detected by fluorography after exposure for 13 [^{35}S-labeled $p21^{v\text{-}H\text{-}ras}$ and p21(GSSKS)] or 42 days. The positions of the precursor (upper) and mature (lower) forms of the parental $p21^{v\text{-}H\text{-}ras}$ (arrows) and p21(GSSKS) proteins (bars) are indicated. Lane 1, ^{35}S-labeled Harvey $p21^{v\text{-}H\text{-}ras}$; lane 2, ^{35}S-labeled p21(GSSKL); lane 3, p21(GSSKL) from [^3H]myristic acid-labeled cells; lane 4, ^{35}S-labeled p21(GSSKS); lane 5, p21(GSSKS) from [^3H]myristic acid-labeled cells; lane 6, p21(GSSKS) from [^3H]myristic acid-labeled cells treated with emetine; lane 7, hydroxylamine-treated p21(GSSKS) from [^3H]myristic acid-labeled cells.

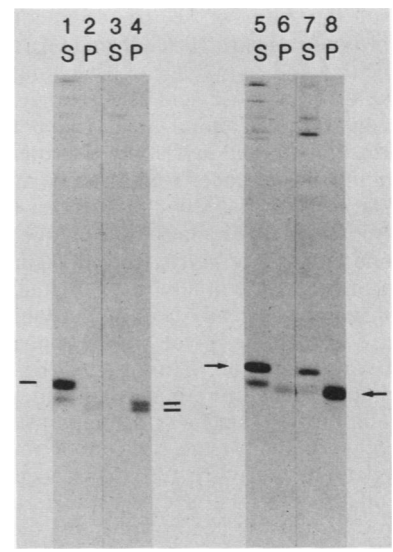

FIG. 2. Comparison of maturation and membrane-binding of p21(GSSKS) and $p21^{v\text{-}H\text{-}ras}$. Four cultures were labeled for 30 min with ^{35}S-TransLabel (200 μCi/ml). One dish of each cell type was then chased for an additional 30 min in medium containing the normal amounts of methionine and cysteine. All the cultures were then separated into cytosolic (S100 [S]) and particulate (P100 [P]) fractions by hypotonic lysis and centrifugation. $p21^{ras}$ proteins were isolated by immunoprecipitation with antibody 146-3E4 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fluorographic exposure was for 14 days. p21(GSSKS) (bars) and $p21^{v\text{-}H\text{-}ras}$ (arrows) are indicated. Lane 1, S100, p21(GSSKS) labeled 30 min; lane 2, P100, p21(GSSKS) labeled 30 min; lane 3, S100, p21(GSSKS) after chase; lane 4, P100, p21(GSSKS) after chase; lane 5, S100, $p21^{v\text{-}Ha\text{-}ras}$ labeled 30 min; lane 6, P100, $p21^{v\text{-}Ha\text{-}ras}$ labeled 30 min; lane 7, S100, $p21^{v\text{-}Ha\text{-}ras}$ after chase; lane 8, P100, $p21^{v\text{-}Ha\text{-}ras}$ after chase.

the extent of p21(GSSKS) myristylation. Thus, the attachment of a myristyl group had little effect on maturation or membrane association of $p21^{v\text{-}H\text{-}ras}$. Further experiments are under way to determine if the myristyl group on p21(GSSKS) can functionally replace the C-terminal palmitate which is required for transformation by $p21^{v\text{-}ras}$ (32).

Myristylation of the p21(GSSKS) protein is incomplete. The extent of p21(GSSKS) myristylation was examined by comparing the incorporation of [^{3}H]myristic acid into p21(GSSKS) and two other myristylated proteins, the Moloney murine leukemia virus structural protein precursor $Pr65^{gag}$ (11), and the alpha subunit of two GTP-binding regulatory proteins associated with inhibition of adenyl cyclase, $G_{i\alpha}1$ and $G_{i\alpha}2$ (3, 17, 22). $Pr65^{gag}$ had been shown by fast atom bombardment-mass spectrometry to contain 1 mol of myristic acid per mol of protein (11). Moreover, previous work had shown that, in NIH 3T3 cells which contained both $Pr65^{gag}$ and $p60^{v\text{-}src}$, the full-length $p60^{v\text{-}src}$ protein was also myristylated fully (4). Cells harboring the p21(GSSKS) protein were first infected with Moloney murine leukemia virus to induce the production of $Pr65^{gag}$. All three proteins were then immunoprecipitated sequentially from a single lysate of Moloney murine leukemia virus-infected cells labeled with [^{3}H]myristic acid (Fig. 3). The abundance of each protein was determined by immunoprecipitation in parallel from ^{35}S-labeled cells. The amounts of [^{3}H]myristic acid or ^{35}S incorporated were quantified by densitometry of fluorograms. The number of methionine plus cysteine residues in $G_{i\alpha}1$ and $G_{i\alpha}2$ was taken as 18 (12); in $Pr65^{gag}$, it was 8 (26), and in $p21^{v\text{-}H\text{-}ras}$, it was 10 (27). A comparison of the ratio of ^{3}H to ^{35}S incorporated into p21(GSSKS) to the ratios for $Pr65^{gag}$ and $G_{i\alpha}$ indicated that p21(GSSKS) contained an average of 27% (range, 10 to 38%; $n = 5$) as much [^{3}H]myristic acid as the other proteins.

From the diversity of amino acids found at the amino termini of already identified myristylated proteins (6, 24, 29) and the multitude of sequences which can be myristylated in vitro (28–30), it is clear that Gly-2-X-X-X-Ser-6 cannot be a general acylation signal. The lack of a unique set of amino acids that operates as a myristylation signal will make identification of other potentially myristylated proteins difficult. One protein has already been found which possesses both glycine 2 and serine 6 yet which appears not to be myristylated within the cell. Transducin, the GTP-binding protein which interacts with retinal rhodopsin, was analyzed chemically, and no fatty acid was detected (3). However, a synthetic peptide analog of the transducin amino terminus could be myristylated in vitro (29). This suggests that the presence of or access to the myristyl transferase may limit myristylation of otherwise acceptable substrates in vivo.

p21(GSSKS) is one of the first examples of a protein that is only partially myristylated. This does not reflect an intrinsic problem with myristylation of a *ras* protein, as the naturally occurring Rasheed $p29^{gag\text{-}ras}$ protein is myristylated as a result of the $p15^{gag}$ sequences at its amino terminus (21; J. E. Buss, unpublished results). A recent study has also shown that mutants of $p60^{v\text{-}src}$ with asparagine following serine 6 were myristylated only 20% as well as wild-type protein with a lysine at that position (15).

Studies of myristate attachment have previously been limited by the apparent lack of enzymes or other mechanisms to remove or regulate the extent of myristylation of a protein. The identification of a sequence (GSSKSV) which produces a limited extent of myristylation should, in fact, be useful. Complete lack of myristylation of $p60^{v\text{-}src}$ produces a protein which fails to transform cells (8, 13, 14). It may prove possible to control the spectrum of events that constitute transformation by regulating the extent of $p60^{v\text{-}src}$ myristylation. The introduction of mutations (particularly at position 7) into other proteins that are normally myristylated, retaining the framework of glycine 2 and serine 6, would be the first available method for regulating this type of acylation in order to produce graded amounts of myristyl protein. This previously unrecognized opportunity should facilitate analysis of lipid modification and its effects on the function of other myristyl proteins.

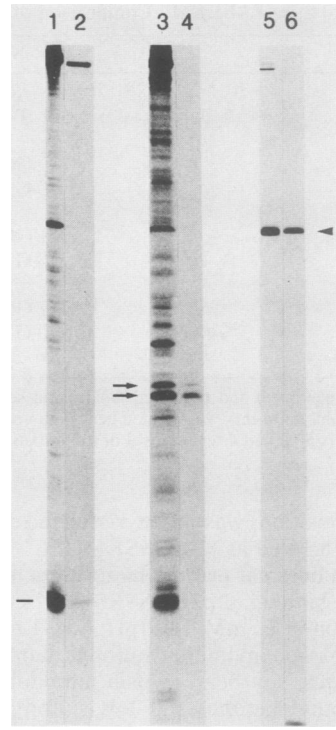

FIG. 3. Comparison of the myristylation of p21(GSSKS) with $Pr65^{gag}$ and $G_{i\alpha}$. p21(GSSKS)-containing cells infected with Moloney murine leukemia virus were labeled for 4 h with either ^{35}S-TransLabel (100 μCi/ml) or [^{3}H]myristic acid (1 mCi/ml). Proteins were isolated sequentially by immunoprecipitation from a single lysate with antibody 146-3E4 [p21(GSSKS), bar], antibody A569 (two forms of $G_{1\alpha}$, arrows), or anti-$p15^{gag}$ serum ($Pr65^{gag}$, arrowhead). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were detected by fluorographic exposure for 3 days for $Pr65^{gag}$ or 35 days for $G_{i\alpha}$ or p21(GSSKS). Care was taken to adjust sample volumes so that a single fluorographic exposure was scanned for the [^{3}H]myristic acid- or ^{35}S-labeled forms of each protein. Twice as much of the $G_{i\alpha}$ samples was loaded as of the other two proteins. Peaks from the two forms of $G_{i\alpha}$ were not resolved completely and were analyzed together. Lane 1, ^{35}S-labeled p21(GSSKS); lane 2, [^{3}H]myristic acid-labeled p21(GSSKS); lane 3, ^{35}S-labeled $G_{i\alpha}$; lane 4, [^{3}H]myristic acid-labeled $G_{i\alpha}$; lane 5, ^{35}S-labeled $Pr65^{gag}$; lane 6, [^{3}H]myristic acid-labeled $Pr65^{gag}$.

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ADDENDUM IN PROOF

Preliminary experiments indicate that myristylation of a p21(GSSKSK) mutant is two- to threefold greater than that of p21(GSSKS), which has a valine at position 7.

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