Amino Terminal Myristylation of the Protein Kinase p60*src*, a Retroviral Transforming Protein

Abstract. *The transforming protein of Rous sarcoma virus, p60*src, *was shown to be acylated at its amino terminus with the long-chain fatty acid myristic acid by isolation of a tryptic peptide with the following structure: myristylglycylserylseryllysine. The occurrence of this unusual posttranslational modification in the cyclic adenosine monophosphate–dependent protein kinase and in several transforming protein kinases of mammalian retroviruses suggests that myristylation of the amino terminal glycyl residue may be critical for the function of certain proteins related to cell transformation and growth control.*

Myristylation was discovered in retroviral structural proteins (*1*) and in two cellular proteins (*2*). In each case the myristyl moiety was found in an amide linkage with the amino terminal glycine residue of the modified protein. The specific occurrence of this rare fatty acid led to speculation that the myristyl moiety was an important structural feature that made proteins capable of recognition by specific cellular membrane-bound receptors (*1*). The two cellular proteins known to be modified in this way are the catalytic subunit of the cyclic adenosine monophosphate (AMP)–dependent protein kinase and calcineurin B, a component of a calmodulin-binding phosphatase (*3*). Furthermore, the myristylated retroviral *gag* protein is found in mammalian *gag-onc* transforming proteins (*4*). These observations led to the further proposal (*1*, *4*) that important regulatory phosphorylation reactions involved in the complex mechanism of growth control would occur at these hypothetical receptor sites for myristylated proteins. Thus targeting by myristylation of *gag-onc* fusion proteins to these sites might be a prerequisite for transformation.

Not all retroviral transforming proteins are products of *gag-onc* fusions. The protein p60*src*, the most intensively studied oncogene product and the first oncogene protein to be identified, contains no retroviral *gag* sequence (*5*, *6*). Since it had been shown to incorporate fatty acid label in the NH_2-terminal region (*7*), and since p60*src* is "blocked" to Edman degradation (suggesting an NH_2-terminal modification) (*8*), we investigated the incorporation of [^{3}H]myristic acid into p60*src*. Here we show that p60*src*, like the mammalian *gag-onc* transforming proteins, is also an *N*-myristylated protein.

The p60*src* was labeled in vivo with [^{3}H]myristate and isolated by immunoprecipitation, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and excision from the gel. The incorporated fatty acid was liberated by hydrolyzing the gel slice in 6*N* HCl, and then was extracted with ether and identified by reversed-phase thin-layer chromatography (*4*). Over 95 percent of this recovered ^{3}H-labeled material comigrated with authentic [^{3}H]myristic acid [migration of myristate relative to solvent (R_F myristate = 0.47, R_F palmitate = 0.40, R_F stearate = 0.30). The myristate attachment site was localized to the NH_2-terminal region of the protein by analysis of V8 protease digests of p60*src* (Fig. 1, lanes 3 and 4). Myristate was not found in the carboxyl terminal fragment V2, but occurred in the NH_2-terminal fragment V1, and in two other digestion fragments (V3 and V4) known to include the amino terminus (*5*). In addition, genetic evidence from deletion mutants suggests that, of the NH_2-terminal 264 amino acids (fragment V1), the sequence specifying the myristate attachment lies within the first 14 residues (*9*).

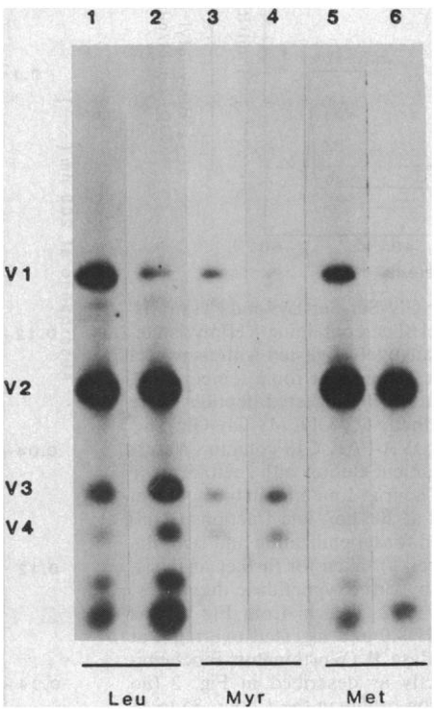

Fig. 1. Partial V8 proteolytic map of p60*src*. The p60*src* was immunoprecipitated from Schmidt-Ruppin A transformed cells labeled for 4 hours with [^{3}H]leucine, [^{3}H]myristate, or [^{35}S]methionine as described (*9*). Excised p60*src*-containing bands were treated with V8 protease during re-electrophoresis. Lanes 1, 3, and 5 represent digestion with 50 ng of V8 protease; lanes 2, 4, and 6 represent digestion with 500 ng V8 protease.

The deduced NH_2-terminal sequence of p60*src* is Met-Gly-Ser-Ser-Lys (*5*, *6*), and the partial V8 map of p60*src* confirmed that the initiator methionine residue was no longer present (Fig. 1, lanes 5 and 6). Therefore, tryptic digestion would be expected to produce an NH_2-terminal tetrapeptide that would be myristylated at the NH_2-terminal glycine residue if p60*src* is an NH_2-myristylated protein. We obtained three peptides for use as chromatographic standards: Myristyl (My)-Gly-Ser-Ser-Lys, My-Gly-Gln-Thr, and My-Gly (*10*). Their high-performance liquid chromatography (HPLC) elution positions are shown in Fig. 2. To produce other peptides as chromatographic standards, we digested synthetic My-Gly-Ser-Ser-Lys with carboxypeptidase A and obtained four peptides separable by reversed-phase HPLC (rpHPLC) (Fig. 3C). Amino acid analysis (Table 1) identified each peak in the chromatogram. An absorbance peak of uncertain origin, which contained no amino acids, eluted just before My-Gly in the chromatogram. My-Gly generated by enzymic digestion co-eluted with the authentic My-Gly. Amino acid compositions of all peptides were in exact agreement with the structures expected from synthesis.

A gel slice containing [^{3}H]myristate-labeled p60*src* was exhaustively digested with trypsin (Fig. 2). The solubilized material was removed from the gel, mixed with synthetic My-Gly-Ser-Ser-Lys, My-Gly-Gln-Thr, and My-Gly in 6*M* guanidinium hydrochloride, and the mixture was separated (Fig. 2) by rpHPLC. More than 80 percent of the radioactively labeled material present in the gel slice was recovered comigrating with the synthetic My-Gly-Ser-Ser-Lys, which was well resolved from synthetic My-Gly-Gln-Thr, and My-Gly.

To prove that the radioactive peptide (Fig. 2, peak a) obtained from p60*src* was indistinguishable from the co-eluting synthetic My-Gly-Ser-Ser-Lys, we digested the mixture with carboxypeptidase B [an enzyme specific for COOH-terminal basic amino acids (*11*)] and separated the products by rpHPLC. Radioactively labeled material was recovered in the two major absorbance peaks, one occurring at the elution position of undigested My-Gly-Ser-Ser-Lys and the other occurring at the elution position of

Table 1. Amino acid analysis of synthetic peptides. Molar ratios, normalized to 1.0 for glycine, after 3 hours of hydrolysis at 130°C. Serine values are corrected for the 20 percent destruction of this labile amino acid which occurs during hydrolysis.

	Myristyl-Gly-Ser-Ser-Lys	Myristyl-Gly-Ser-Ser	Myristyl-Gly-Ser	Myristyl-Gly
Gly	1.0	1.0	1.0	1.0
Ser	2.0	1.9	1.1	0
Lys	0.92	0.03	0	0

My-Gly-Ser-Ser (Fig. 3, A and C). Further evidence for identity was obtained by combining peaks a and b (Fig. 3A) and continuing digestion of the radioactive p60*src* peptides with carboxypeptidase A (an enzyme specific for neutral amino acids). The resulting peptide mixture was again separated by rpHPLC (Fig. 3B) and comigration of radioactivity from the p60*src* peptide and absorbance from digestion products of the synthetic My-Gly-Ser-Ser-Lys was again observed. Each of the four peptides produced from the [^{3}H]myristate-labeled p60*src* comigrated with a peptide derived from the synthetic My-Gly-Ser-Ser-Lys. My-Gly was produced in highest yield.

It is significant that four peptides were generated during the digestion (Fig. 3B) because it shows that carboxypeptidase A had cleaved all three peptide bonds, including the Gly-Ser bond. Dipeptides with free amino groups are poor substrates for carboxypeptidase A but become good substrates if the NH_2-terminus is acylated (*12*). Thus if the Gly were not acylated, carboxypeptidase A would produce only three peptides instead of the four observed. This is additional evidence that the myristate is linked to the glycine. An ester linkage seems unlikely since myristate-labeled p60*src* bands in unfixed gels were resistant to 1 hour 37°C treatment in either 1*M* NH_2OH-HCl, *p*H 6.6, or 0.1*M* KOH in 40 percent methanol (*13*). This observation and the fact the p60*src* is "blocked" to Edman degradation (*8*) are both consistent with the NH_2-terminal amide linkage.

Our data show that p60*src* is myristylated at its NH_2-terminus exactly as in other known myristylated proteins. Recently, NH_2-terminal deletion mutants of p60*src* have been constructed (*9*). These mutant proteins are still functional kinases in vitro and in vivo but fail to

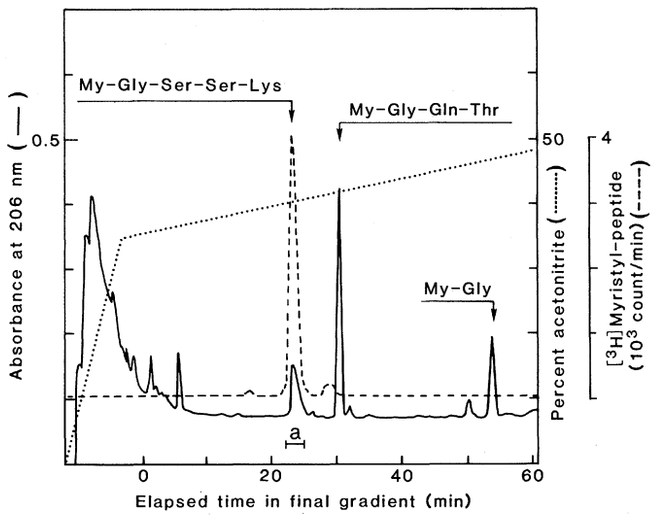

Fig. 2 (left). Cochromatography of synthetic Myr-Gly-Ser-Ser-Lys and [^{3}H]myristylate-labeled tryptic peptide from p60*src*. A gel slice containing [^{3}H]myristate-labeled p60*src* was obtained as in Fig. 1 and exhaustively digested with trypsin (1 μg per milliliter of 0.05*M* $NaHCO_3$, *p*H 8.8, for 72 hours at room temperature, with an additional 1 μg added at 24 and 48 hours). The digested peptides were removed from the gel fragments, mixed with synthetic My-Gly, My-Gly-Gln-Thr, and My-Gly-Ser-Ser-Lys and injected onto a NOVA-PAK C18 column (Waters Associates). This was followed by a two-stage gradient elution with acetonitrile (0 to 35 percent in 10 minutes, 35 to 48 percent in 1 hour at 1 ml/min) in 0.05 percent trifluoroacetic acid. The effluent was monitored at 206 nm, and fractions (1 ml) were collected. Portions (250 μl) were withdrawn for lyophilization and scintillation counting. The bar indicates the fraction (peak a) taken for further analysis.

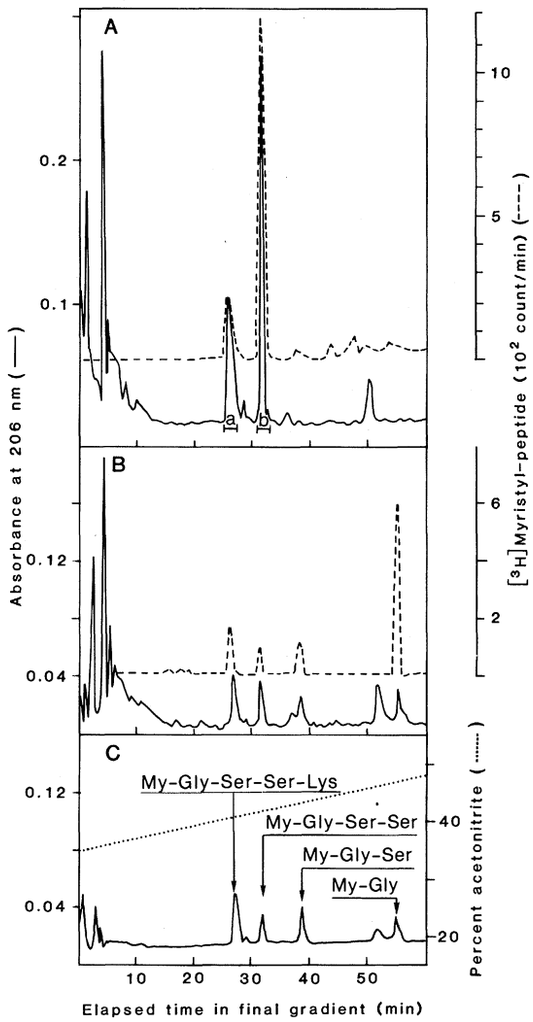

Fig. 3 (right). Analysis of My-Gly-Ser-Ser-Lys by carboxypeptidase digestions. (A) Carboxypeptidase B digest of peak a of Fig. 2. Peak a from Fig. 2 was lyophilized and dissolved in 0.01*M* tris-HCl, *p*H 8.0 and digested overnight at room temperature with 1.5 μg of carboxypeptidase B (Worthington Biochemicals). It was then separated by rpHPLC exactly as described in Fig. 2 (no synthetic peptide was added). Shown is the elution profile in the 1 hour, 35 to 48 percent acetonitrile gradient. The final gradient shape, common to all three panels, is shown in (C). Fractions were collected each minute, and portions (20 percent) were used for scintillation counting. (B) Carboxypeptidase A digest of peaks a and b of (A). Peaks a and b from (A) were pooled, lyophilized, and dissolved in 0.01*M* tris-HCl, *p*H 9.0, and digested overnight at room temperature with carboxypeptidase A (3.5 μg/ml, Worthington Biochemicals). They were then separated by rpHPLC as described in (A). Fractions were collected each minute and lyophilized for scintillation counting. (C) Carboxypeptidase A digest of synthetic My-Gly-Ser-Ser-Lys. The unlabeled, synthetic myristylated tetrapeptide was digested with carboxypeptidase A and separated by rpHPLC as described in (A). The 35 to 48 percent linear acetonitrile gradient was also common to the experiments described in (A) and (B). Amino acid compositions of four peaks are given in Table 1; the fifth peak did not contain detectable amino acids.

incorporate myristate, are no longer localized to membranes and, most importantly, do not transform the cells. The behavior of these constructed mutants is consistent with the interpretation that the mutant p60*src* protein now lacking myristate may be no longer capable of finding the sensitive membrane-bound targets to induce transformation. Similar experiments deleting the myristate acceptor site in the *gag-abl* protein have shown that myristate may have a role in transformation in this system as well (*14*).

Whether there are specific inner membrane receptors for myristylated proteins as suggested here remains to be established. Certain observations suggest that interplay of myristylated proteins at the membrane is important in transformation and growth control. For example, the activity of type I cyclic AMP–dependent kinase has been shown to be depressed fivefold in *src*-transformed cells (*15*), and the cyclic AMP–dependent kinase regulatory subunit can be a substrate for calcineurin (*16*). Myristylation may prove useful for characterizing the cellular sites where these interactions take place, and for studying the physiology and biochemistry of transforming proteins and their proto-oncogene homologues.

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10. Myristyl (My)-Gly-Ser-Ser-Lys and My-Gly-Gln-Thr were prepared by solid-phase Merrifield synthesis methods (T. Copeland, Frederick Cancer Research Facility, National Cancer Institute) and synthetic My-Gly was the gift of V. E. Marquez (Laboratory of Medicinal Chemistry and Biology, National Cancer Institute). Amino acid analysis of My-Gly yielded only glycine, and nuclear magnetic resonance and infrared spectra were consistent with the structure.
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Antibodies to Peptides Detect New Hepatitis B Antigen: Serological Correlation with Hepatocellular Carcinoma

Abstract. *The expression of a previously unidentified gene product, encoded by the hepatitis B virus (HBV) genome, has been achieved with a recombinant SV40 expression vector. Antibodies against synthetic peptides representing defined regions of this protein were used to screen cells infected with recombinant virus as well as tissues naturally infected with HBV. A 24,000-dalton protein (p24) was detected in cells infected with recombinant virus and a 28,000-dalton protein (p28) was detected in tissues infected with HBV. The peptides or recombinant-derived protein were used as antigens to screen sera from individuals infected with HBV. Specific antibodies were detected predominantly in sera from patients with hepatocellular carcinoma. The presence of p28 in tissues infected with HBV and the appearance of specific antibodies in infectious sera establish the existence of an additional marker for HBV infection.*

Analysis of the viral gene products encoded by the hepatitis B virus (HBV) has been hampered by the lack of a permissive tissue culture system and the narrow host range demonstrated by HBV. The cloning of the HBV genome (*1*) has provided a means by which these problems can be circumvented. DNA sequence analysis of these cloned HBV genomes (*2, 3*) identified the genes encoding the surface and core antigens and, in addition, revealed two open reading frames (denoted P and X) on the L strand (*4*). It has been suggested that the endogenous DNA polymerase found associated with core particles is encoded by region P, since the translation product of this sequence is homologous to conserved regions of retrovirus *pol* gene products (*5*). Region X represents an open reading frame for which a protein product has yet to be assigned. Definition of the hepatitis B surface antigen (HBsAg), core antigen (HBcAg) and "e" antigen (HBeAg) on the genome has allowed the production of these classical HBV markers in eukaryotic expression systems. In addition to providing an alternative source from which to obtain HBV gene products, these techniques can be used to generate large quantities of materials for an HBV vaccine (*6*). We now report the expression of the HBV X gene product as well as its detection in tissues infected with HBV with antibodies of predetermined specificity. We also detected circulating antibodies specific for the X protein in human serum samples infected with HBV. Together, these findings establish the existence of a new marker for HBV infection.

Hepatitis B virus DNA was cloned into plasmid vector pBR322 at the Bam HI restriction site, resulting in a full genome interrupted in the pre-S region and retaining both Bam HI sites (Fig. 1A). One Bam HI fragment contained the HBsAg coding sequence [1350 base pairs (bp)] while the other fragment contained 94 percent of the X coding sequence and all of the core gene (1850 bp). Expression of the S gene was accomplished as described (*7*). The DNA sequence containing the X gene (1850-bp Bam HI fragment) was ligated to a pBR322-SV40 cloning vector (pBRSV) at the Bam HI site. The plasmid sequences in pBRSVHBV permitted the replication of this hybrid DNA in *Escherichia coli* prior to transfection into eukaryotic cells. A restriction enzyme digest with Hae II removed all of the plasmid sequences and positioned the HBV fragment in the sense orientation to the SV40 late promoter (SVHBV-3). Construction of the expression vector SVHBV-3 removed the first 66 bp of the putative X gene, including the methionine initiation codon ATG. We predicted that the X sequences present in the construct (Fig. 1B, amino acid residues 23 to 154) would fuse with the SV40 structural protein VP2 sequences present in the vector.