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NOTES

Posttranslational Processing of p21 *ras* Proteins Involves Palmitylation of the C-Terminal Tetrapeptide Containing Cysteine-186

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The p21 proteins of *ras* oncogenes are synthesized as precursors in the cytosol. After processing, which involves acylation, the products are associated with the plasma membrane in eucaryotic cells. The p21 overproduced in *Escherichia coli*, however, is not processed by acylation. A synthetic tetrapeptide of the p21 C terminus is used to identify the acylation site in eucaryotic p21 as cysteine-186. The same peptide of bacterial p21 is not acylated. Although p21 of Harvey murine sarcoma virus-transformed NRK cells can be metabolically labeled with either [^{3}H]palmitate or [^{3}H]myristate, the lipid moiety of the hydrophobic peptide is identified as palmitic acid. We suggest that the enzymatic mechanism for p21 palmitylation may be different from N-terminal myristylation of many other membrane proteins.

The viral transforming genes of Harvey murine sarcoma virus (Ha-MuSV) and Kirsten murine sarcoma virus (Ki-MuSV) encode the 21,000-dalton p21 *ras* proteins (5, 7, 8, 29, 31, 32, 35). A family of structurally highly conserved p21 proteins is encoded by cellular homologs of the viral *ras* genes (v-*ras*). These cellular homologs activated by point mutations have been detected in a variety of human neoplasms. Posttranslational processing of p21 appears to be a common pathway for the synthesis of all p21 *ras* proteins (30, 34). Sefton et al. (26) demonstrated acylation of p21 in the processed products. The importance of p21 acylation to its transforming function was most clearly demonstrated by Willumsen et al. (36, 37) in a series of genetic mutations located near the p21 C terminus. Studies of these mutants indicate that cysteine-186 is essential, although not sufficient, for p21 processing. The exact role of cysteine-186 in p21 processing is uncertain from the mutant studies; however, we demonstrate here direct chemical evidence indicating that cysteine-186 is the palmitylation site. We suggest that the sequence surrounding this site may be required for recognition by cellular palmitylation enzymes.

Figure 1 shows a comparison of tryptic peptide maps of pro-p21 and p21 labeled with [^{35}S]cysteine. It is clear that there is a peptide (indicated by an arrow) in pro-p21 that is absent in the processed p21 because of either modification or cleavage during posttranslational processing. To determine the site of acylation more directly, we took advantage of the abundant source of p21 overproduced in *Escherichia coli* as an equivalent of pro-p21 (14). The p21 overproduced in *E. coli* was not labeled by [^{3}H]palmitate (Fig. 2A), as is p21 in Ha-MuSV-transformed NRK (Ha-NRK) cells. p21 in *E. coli* is not processed as it is in mammalian cells, as revealed by slight mobility changes in short pulse-labeling experiments (30). There was no change in the mobility of *E. coli* p21 during labeling periods of 1.5, 5, 10, 30, and 120 min (data not shown). Therefore, *E. coli* p21 serves as a convenient equivalent of pro-p21 for comparison of its peptide map. *E. coli* p21 is a fusion protein (24,000 daltons), with 4 N-terminal residues of v-*ras* p21 replaced by 14 residues from the expression vector, but there is no cysteine residue in these sequences to complicate the labeling experiments.

An Ha-NRK cell line was grown in 60-mm tissue culture dishes (31). After reaching confluence, cells were labeled at 37°C for 18 h with 5 ml of amino acid-free Dulbecco-Vogt medium supplemented with 2% dialyzed fetal calf serum and containing 2 mCi of L-[^{35}S]cysteine (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and 5 mCi of 9,10(*n*)-[^{3}H]palmitate (50 Ci/mmol; Amersham). The latter isotope in toluene was dried under a stream of N_2 and was dissolved in 50 μl of dimethyl sulfoxide before mixing with the medium. p21 was isolated from cell lysates by immunoprecipitation with a monoclonal antibody (YA6-172), and the precipitated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (28, 31, 32). After autoradiography, the wet gel pieces containing the p21 band were excised and eluted as described previously (28) with 100 μg of unlabeled purified p21 overproduced in *E. coli* as a carrier (11). To obtain [^{35}S]cysteine-labeled *E. coli* p21, bacterial cells carrying the plasmid pJLcIIrasI (constructed by Lautenberger et al. [14]) were grown at 32°C and were induced by shifting the temperature to 41°C as previously described (14). After SDS-PAGE, p21 was eluted as above. Free thiol groups of p21 were carboxymethylated with iodoacetate after reduction with dithiothreitol by the procedure described by Allen (1), but with proteins in 1% SDS instead of guanidine hydrochloride (Fig. 3). In most other experiments, p21 was not chemically treated to preserve the attached lipid. After dialysis and lyophilization, the labeled proteins (120,000 cpm of *E. coli* p21; 80,000 cpm of Ha-MuSV p21), with 100 μg of carrier p21, were digested with 18 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Sigma Chemical Co., St. Louis, Mo.) in 100 μl of 0.2 M sodium phosphate

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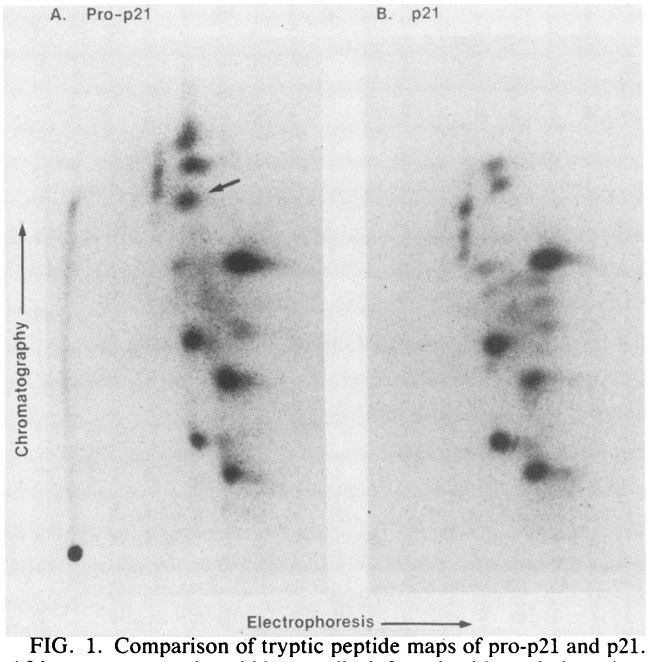

FIG. 1. Comparison of tryptic peptide maps of pro-p21 and p21. African green monkey kidney cells infected with a simian virus 40-Harvey-*ras* recombinant virus (10) were labeled with [^{35}S]cysteine for 30 min for pro-p21 or 20 h for p21. After immunoprecipitation and SDS-PAGE, the p21 and pro-p21 bands were eluted. The two-dimensional tryptic peptide map was prepared as previously described (8, 31). The arrow indicates the unique peptide in pro-p21. (A) pro-p21; (B) p21.

buffer (pH 8.0) for 11 h at 37°C. The reaction mixture was diluted to 500 μl with 10 mM phosphoric acid before injection into a reversed-phase high-pressure liquid chromatography (HPLC) column (μBondapak Phenyl, 3.9 mm by 30 cm; Waters Associates, Inc., Milford, Mass.). Peptides were eluted with linear gradients of acetonitrile (HPLC grade; Fisher Scientific Co., Pittsburgh, Pa.) in 10 mM phosphoric acid first from 0 to 48% in 90 min and then from 48 to 80% in 20 min at a flow rate of 1 ml/min. The effluent was monitored at 210 nm for the carrier p21, and fractions of each major peak and the interpeak regions (ca. 1 ml) were collected. Differential counting of ^{3}H and ^{35}S was performed in a Beckman liquid scintillation system at window settings of narrow ^{3}H and ^{3}H plus ^{14}C for ^{35}S with samples in Aquassure (New England Nuclear Corp., Boston, Mass.). The counting ratio for the ^{3}H standard was 0.94; for the ^{35}S standard, it was 0.16. All the major peaks in the effluent had a ratio of 0.17, similar to that of the ^{35}S standard. The exception was the peptide *b* peak, indicated in the double-hatched area in Fig. 3B, which had a ratio of 0.8, indicating double labeling by ^{3}H and ^{35}S. HPLC patterns of the unlabeled *E. coli* p21 peptides were very similar (Fig. 3). We noticed a small reproducible [^{35}S]cysteine-labeled peptide of *E. coli* p21, indicated as peptide *a* in Fig. 3A, which was absent in p21 of Ha-NRK cells. (The peptide peak is split in this carboxymethylated sample perhaps because of incomplete reaction with iodoacetate in an SDS-protein micelle. In most tryptic digests without modification, it is seen as a single peak.) Instead, the Ha-MuSV p21 displays a hydrophobic peptide (peptide *b* in Fig. 3B) that eluted at high concentrations of acetonitrile and was double labeled with ^{3}H-fatty acid in addition to [^{35}S]cysteine. To identify these peptides, we synthesized a Cys-Val-Leu-Ser tetrapeptide (CVLS peptide) of the sequence NH_2-Cys-Val-Leu-Ser-COOH, by an automated solid-phase method (18) according to the expected C-terminal sequence of v-*ras*^{H} p21 following the trypsin cleavage site at lysine-185 (7). This CVLS peptide was eluted from HPLC at the position of the unique peptide *a* of *E. coli* p21 as indicated in Fig. 3A. (The CVLS peptide was also carboxymethylated in this experiment.) To further confirm the identity of these peptides, the [^{35}S]cysteine-labeled peptide *a* obtained by HPLC was mixed with synthetic CVLS peptide and was cochromatographed as shown in Fig. 4A. Two sets of experiments were performed by modifying the thiol group of CVLS peptide either with oxidation or carboxymethylation. The oxidized form is shown in Fig. 4A. The ^{35}S-labeled peptide *a* was obtained by HPLC from a trypsin digest of *E. coli* p21 without any prior chemical treatment. After lyophilization, it was mixed with 5 μg of the synthetic CVLS peptide, and the mixture was treated with performic acid according to the method of Beemon and Hunter (2) to oxidize the thiol group. After lyophilization, the peptides were analyzed by the μBondapak Phenyl column eluted with a linear acetonitrile gradient from 0 to 23.3% in 0.05% trifluoroacetic acid (Fisher) in 35 min. The flow rate was 1 ml/min, and 1-ml fractions were collected. The ^{35}S-labeled peptide *a* coeluted precisely with the synthetic CVLS as monitored by A_{210} (Fig. 4A). Similar results were obtained with the carboxymethylated form of peptide *a*,

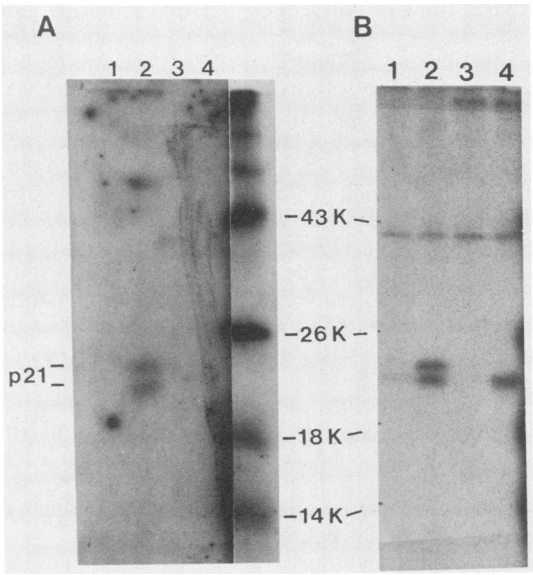

FIG. 2. [^{3}H]palmitate labeling of p21 in Ha-NRK and Ki-MuSV-transformed NRK (Ki-NRK) cells and p21 expressed in *E. coli*. (A) Ha-NRK cells were labeled with [^{3}H]palmitate (1 mCi/ml) for 18 h, and *E. coli* overproducing p21 was labeled for a period of 30 min, beginning 10 min after induction of p21 synthesis. p21 was immunoprecipitated from cell lysates containing 10^{7} trichloroacetic acid-precipitable counts per minute, with a monoclonal antibody against p21 (YA6-172). The precipitated proteins were resolved by SDS-PAGE and were visualized by fluorography. Lanes: 1, Ha-NRK lysate with normal immunoglobulin G; 2, Ha-NRK lysate with antibody; 3, *E. coli* lysate with normal immunoglobulin G; 4, *E. coli* lysate with antibody. (B) Ha-NRK and Ki-NRK cells were labeled with [^{3}H]palmitate and immunoprecipitated as above. Lanes: 1, Ha-NRK lysate with normal immunoglobulin G; 2, Ha-NRK lysate with antibody; 3, Ki-NRK lysate with normal immunoglobulin G; 4, Ki-NRK lysate with antibody. K, Protein size standards (10^{3} daltons).

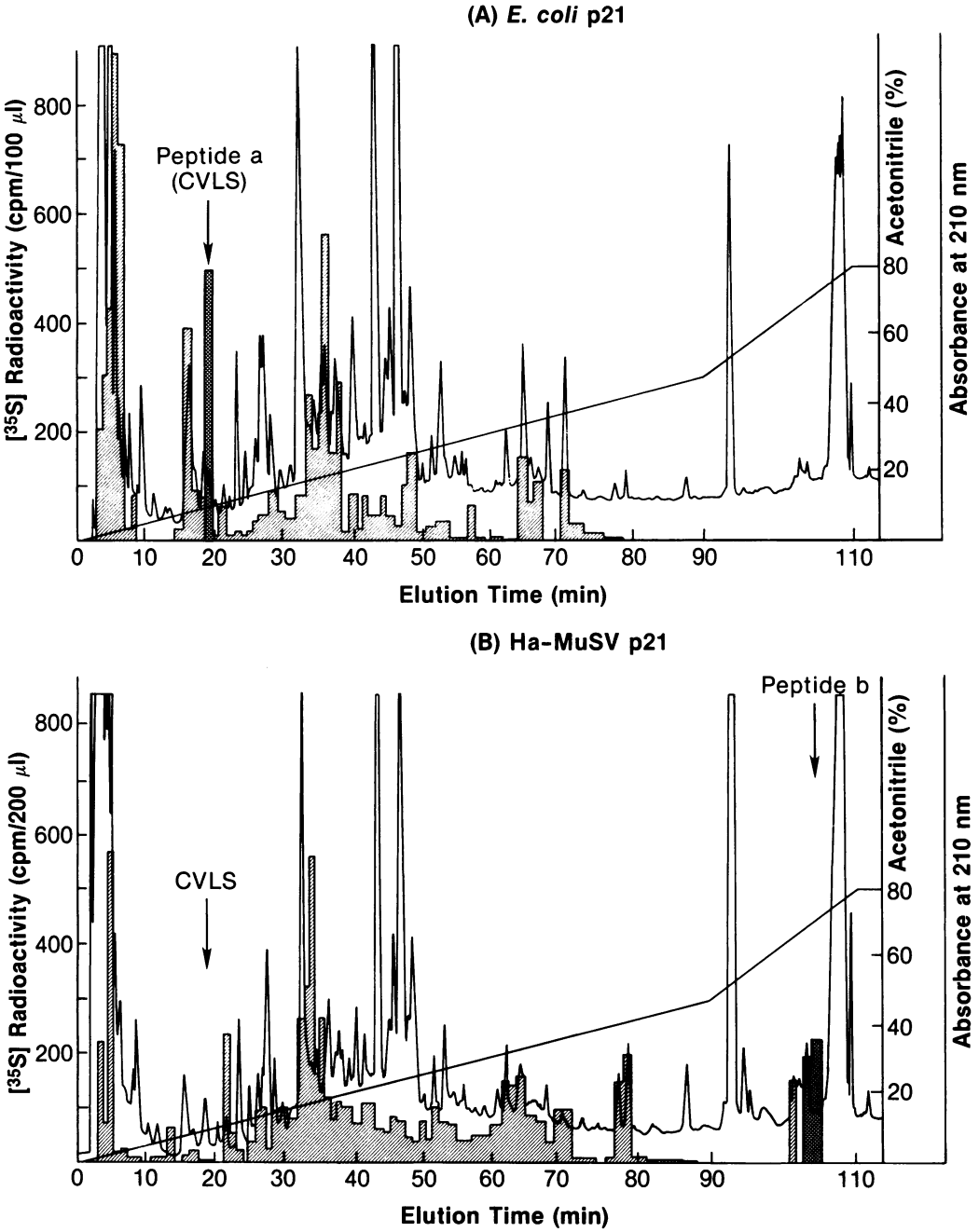

FIG. 3. Comparative HPLC of tryptic peptides of Ha-MuSV p21 and p21 overproduced in *E. coli*. p21 labeled with [^{35}S]cysteine and [^{3}H]palmitate was isolated from Ha-NRK cells or from bacterial cells overproducing p21 by immunoprecipitation with an anti-p21 antibody and SDS-PAGE. The radioactively labeled p21 was mixed with 100 μg of purified unlabeled *E. coli* p21 as a carrier. Tryptic peptides of the carboxymethylated p21 were resolved by HPLC. (A) *E. coli* p21; (B) Ha-MuSV p21. Arrows indicate unique peptides: peptide *a* in *E. coli* cochromatographs with the synthetic tetrapeptide (CVLS), and peptide *b* in Ha-MuSV p21 contains both ^{35}S and ^{3}H radioactive labels. Tracings of A_{210} and blocks of radioactivity of fractions are as shown. The elution position of the synthetic CVLS peptide is indicated by CVLS arrows.

which eluted 3 min later on HPLC. Therefore, we conclude that the unique *E. coli* peptide *a* is the C-terminal tryptic peptide of p21. A radioactive sequencing of [^{35}S]cysteine-labeled peptide *a* also confirmed the expected sequence of Cys-Val-Leu-Ser with most of the radioactivity found in the first cycle of Edman degradation.

The identity of the hydrophobic peptide *b* of Ha-MuSV p21 in the transformed NRK cells is also established by cochromatography with the synthetic CVLS peptide after hydroxylamine cleavage of the fatty acid (Fig. 4B). Peptide *b* was obtained by HPLC from a tryptic digest of Ha-MuSV p21 double labeled with [^{3}H]palmitate and [^{35}S]cysteine. After lyophilization, the material was dissolved in 200 μl of 1 M NH_2OH-HCl, the pH was adjusted to 8.0 with NaOH, and the sample was incubated at 25°C for 8 h. The reaction mixture was extracted with 400 μl of chloroform, and the

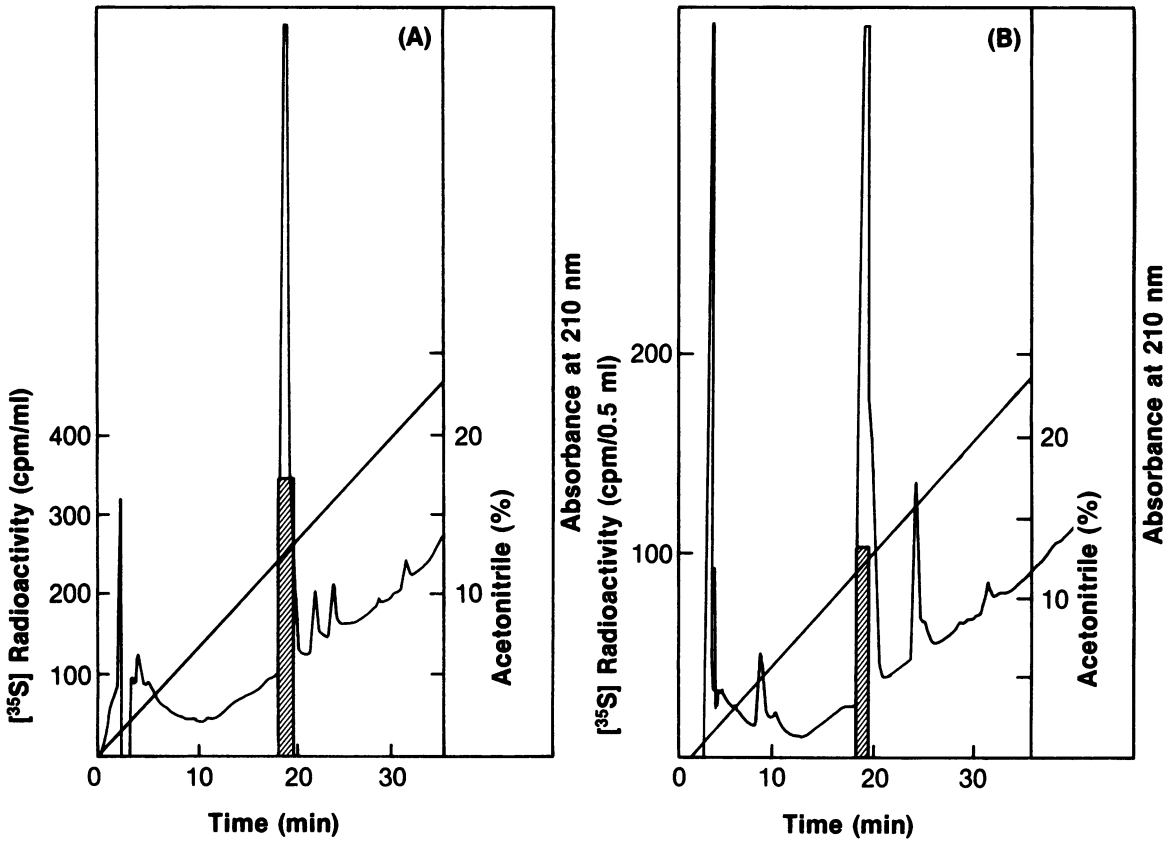

FIG. 4. Cochromatography of the synthetic tetrapeptide CVLS with peptide *a* and the hydroxylamine-treated peptide *b*. (A) The unique peptide *a* of *E. coli* p21 tryptic digest labeled with [^{35}S]cysteine (not carboxymethylated) was mixed with the synthetic peptide (CVLS) and was analyzed by HPLC after performic acid oxidation. (B) The unique hydrophobic peptide *b* of Ha-MuSV double labeled with [^{3}H]palmitate and [^{35}S]cysteine was hydrolyzed with hydroxylamine. After chloroform extraction of fatty acids, the material in the aqueous phase was mixed with the synthetic CVLS peptide and was analyzed by HPLC after performic acid treatment. The hatched areas indicate the radioactivity peaks.

aqueous phase was repeatedly lyophilized. After the addition of 10 μg of synthetic CVLS peptide, the mixture was treated with performic acid as above. The material was lyophilized and dissolved in 500 μl of water and was applied to the HPLC column. The column was eluted with 0 to 60% acetonitrile gradient in 0.05% trifluoroacetic acid, and an area (2 ml) around the CVLS peptide position was pooled. After lyophilization, the mixture was again analyzed by HPLC under the same conditions as in Fig. 4A. This recovered radioactive peptide coeluted with the synthetic CVLS (Fig. 4B). Although the hydrophobic peptide *b* possessed a ^{3}H-to-^{35}S counting ratio of 0.8, the peptide recovered in the aqueous phase had a ratio of 0.18, characteristic of the ^{35}S standard. The ratio for differential counting in the chloroform extract was similar to that of the ^{3}H standard, indicating the presence of ^{3}H-lipid. Thus, we conclude that peptide *b* is an acylated peptide of the p21 product and peptide *a* is the precursor form found in *E. coli* p21.

As p21 in transformed cells can be readily labeled not only by [^{3}H]palmitate, a C_{16} saturated fatty acid, but also by [^{3}H]myristate, which is two carbon atoms shorter than the former (data not shown), we are interested in the nature of the fatty acid attached to peptide *b*. Ha-NRK cells were double labeled with [^{35}S]cysteine (1,000 Ci/mmol; Amersham) and either 9,10(*n*)-[^{3}H]palmitate (50 Ci/mmol; Amersham) or 9,10(*n*)-[^{3}H]myristate (50 Ci/mmol; Amersham) under conditions described above. The hybrophobic peptide *b* was isolated by HPLC after trypsin digestion. The fatty acid was released from the peptide by hydroxylamine cleavage. The reaction mixtures in 200 μl of 1 M NH_2OH-HCl were extracted with 400 μl of chloroform. The chloroform phase was further extracted three times with 200 μl of a chloroform-water mixture (1:10). The organic phase from all extractions was pooled and dried under a stream of N_2. The materials were dissolved in 400 μl of 50% methanol and were applied to the μBondapak Phenyl HPLC column. Elution was with 70% methanol in 0.05% trifluoroacetic acid at 1 ml/min. Fractions of 1.4 ml were collected, and the radioactivity was counted. Palmitic acid and myristic acid standards were well separated. The fatty acid of peptide *b* was palmitic acid irrespective of in vivo labeling either with [^{3}H]palmitate or [^{3}H]myristate. Since some of the fatty acids released from proteins may be in the form of hydroxamates, the elution positions on HPLC of the respective fatty acid hydroxamates were also determined. Hydroxamates were obtained by hydroxylamine cleavage of the model thioester compounds [^{14}C]palmityl and [^{14}C]myristyl coenzyme A (Amersham). Hydroxamates of these fatty acids eluted three fractions earlier than did the corresponding free fatty acids under present HPLC conditions (data not shown). Thus, radioactivity released from peptide *b* appears mostly as free palmitic acid, not as its hydroxamate. This is further confirmed by complete hydrolysis of the ^{3}H-labeled products from peptide *b* with 6 N HCl

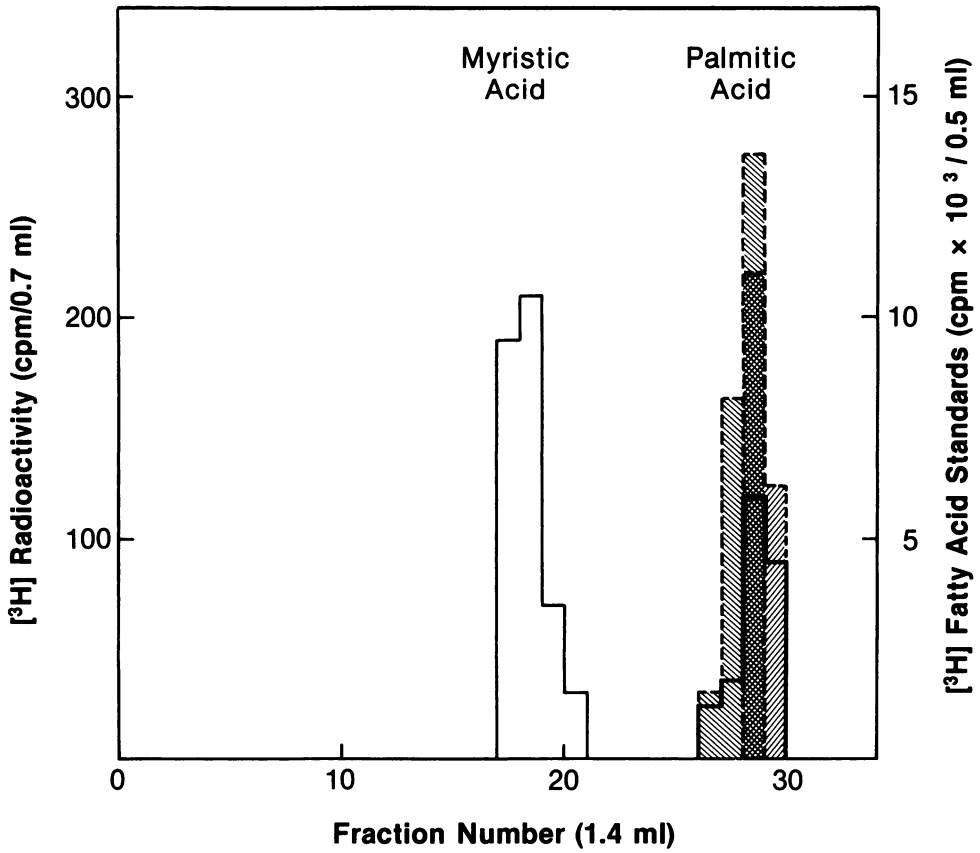

FIG. 5. Identification of the fatty acid associated with the hydrophobic peptide *b*. The fatty acid of Ha-MuSV p21 double labeled either with [^{35}S]cysteine and [^{35}H]palmitate mixture or with [^{3}S]cysteine and [^{3}H]myristate was analyzed with reversed-phase HPLC. The fatty acids were obtained from the chloroform extracts of HPLC-purified peptide *b* after hydroxylamine treatment. The solid blocks depict standard [^{3}H]palmitic acid and [^{3}H]myristic acid. ▨, HPLC profiles of ^{3}H radioactivity from p21 labeled with [^{3}H]palmitate (fractions 29 and 30) performed under the same conditions; ▧, profiles of p21 labeled with ^{3}H-myristate (fractions 27 to 29).

at 110°C for 16 h (4). The latter treatment did not further change the elution position of the ^{3}H products (Fig. 5). Therefore, it is concluded that the fatty acid incorporated into peptide *b* is palmitic acid, not myristic acid. Apparently, these two ^{3}H-fatty acids are metabolically interconvertible in NRK cells.

We conclude that posttranslational processing of p21 involves palmitylation of the C-terminal tetrapeptide of the sequence, Cys-Val-Leu-Ser. Although the chemical evidence does not allow us to rule out palmitylation at the last serine residue, we consider this possibility very unlikely since palmitylation also occurs in p21 of *ras*^{K} (Fig. 2B) and perhaps *ras*^{N} genes, in which the last amino acids are both methionine rather than serine. It is most likely that the palmitic acid moiety is attached to cysteine-186 of p21 molecules through a thioester linkage. Cysteine-186 is conserved in all *ras*-related genes, and the specificity of palmitylation appears to be the cysteine residue 4 amino acid from the C termini. The present studies, however, do not rule out the possibility that p21 C-termini may be heterogeneous with products further processed by proteolytic cleavage after palmitylation at cys-186, since recovery of peptide *b* is not stoichiometric. Preliminary attempts to remove lipid from p21 by treatment with either hydroxylamine or alkaline methanol do not restore p21 mobility on SDS-PAGE to that of pro-p21.

It is interesting to note that although lipidation of p21 is required for membrane association and transformation, the pro-p21 and the p21 overproduced in *E. coli* have all known p21 biochemical activities of GTP/GDP binding (11, 25, 27), autokinase (11, 25, 27), and GTPase (9, 11, 17, 33). Perhaps the p21 molecule has an enzyme domain and another membrane-binding domain at the palmitylation site.

Some oncogene proteins, such as p60 *src*, and several cellular proteins have recently been shown to be myristylated at their N termini through amide linkages (4, 6, 12, 16, 23, 24). Palmitylation of p21 through a thioester linkage near the p21 C terminus may represent a different mechanism for posttranslational lipidation of proteins (13, 15, 19). Many reports have described palmitylation of membrane-associated proteins probably occurring through thioester linkages with internal cysteine residues (3, 20, 21, 22). The exact palmitylation sites of these proteins have not been determined. The present identification of the p21 palmitylation site with a defined sequence may allow direct experimentation to identify and to characterize the enzyme systems for protein palmitylation by using the p21 precursor overproduced in *E. coli* as the enzyme substrate. Studies on these enzyme systems will be very important for understanding posttranslational processing of membrane proteins with critical biological consequences.

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