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# Direct Identification of Palmitic Acid as the Lipid Attached to p21$^{ras}$

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**p21$^{v\text{-}H\text{-}ras}$, the transforming protein of Harvey murine sarcoma virus, contains a covalently attached lipid. Using thin-layer chromatography, we identified the acyl group as the 16-carbon saturated fatty acid palmitic acid. No myristic acid was detected in fatty acids released from in vivo-labeled p21$^{v\text{-}H\text{-}ras}$. The p21$^{v\text{-}K\text{-}ras}$ protein encoded by Kirsten sarcoma virus was also palmitylated. The processing and acylation of p21$^{v\text{-}K\text{-}ras}$ however differed from that of p21$^{v\text{-}H\text{-}ras}$. Three forms of [$^3$H]palmitic acid-labeled p21$^{ras}$ proteins were detected in Kirsten sarcoma virus-transformed cells. This contrasted with Harvey sarcoma virus, in which two forms of p21$^{v\text{-}H\text{-}ras}$ contained palmitic acid. Analysis by partial proteolysis of p21$^{v\text{-}H\text{-}ras}$ labeled with [$^3$H]palmitic acid suggested that all of the lipid found in intact p21$^{v\text{-}H\text{-}ras}$ was located in the C-terminal region. On sodium dodecyl sulfate-polyacrylamide gels, p21$^{v\text{-}H\text{-}ras}$ labeled with [$^3$H]palmitic acid migrated slightly ahead of the majority of p21$^{v\text{-}H\text{-}ras}$. Of the mature forms of p21$^{v\text{-}H\text{-}ras}$, apparently only a subpopulation contains palmitic acid.**

The genome of vertebrates contains at least three *ras* proto-oncogenes, designated c-H-*ras*, c-K-*ras*, and c-N-*ras*. These genes have highly related but distinct nucleotide sequences (7, 11, 38, 39, 43). All three cellular *ras* genes have pronounced oncogenic potential. c-H-*ras* and c-K-*ras* derive their names from the fact that they give rise to the oncogenes found in Harvey sarcoma virus (HaSV) and Kirsten sarcoma virus (8, 10). c-N-*ras* was identified as a dominant transforming gene of a human neuroblastoma cell line (39, 41).

Vertebrate *ras* genes encode proteins of approximately 21,000 daltons, termed p21$^{ras}$ (10, 19, 37). Both cellular p21$^{c\text{-}ras}$ and viral p21$^{v\text{-}ras}$ proteins bind GTP with high affinity (23, 31, 40) and possess an activity which hydrolyzes this nucleotide (14, 22, 24). The biochemical characteristics and amino acid sequences of p21$^{c\text{-}ras}$ and p21$^{v\text{-}ras}$ resemble those of other GTP-binding proteins, the alpha subunits of the nucleotide regulatory (G) proteins of adenylate cyclase and transducin (17, 20). In yeast cells, the *ras* protein homologues modulate adenylate cyclase activity (42). It is not known if the vertebrate p21$^{ras}$ proteins serve a related function.

p21$^{v\text{-}H\text{-}ras}$ is bound to the cytoplasmic face of the plasma membrane (34, 45). This binding is important for the oncogenic activity of p21$^{v\text{-}H\text{-}ras}$. A series of mutant v-H-*ras* genes constructed by Willumsen and co-workers (46, 47) demonstrate that mutants which encode soluble p21$^{v\text{-}H\text{-}ras}$ proteins also lack transforming activity.

Membrane-bound forms of p21$^{v\text{-}H\text{-}ras}$ contain a covalently attached fatty acid (34). This lipid may play a role in the binding of p21$^{v\text{-}H\text{-}ras}$ to membranes, because both the soluble mutant proteins and the soluble precursor to p21$^{v\text{-}H\text{-}ras}$ are not acylated (34, 36, 44, 46). Although the lipid in p21$^{v\text{-}H\text{-}ras}$ was originally discovered because the protein could be labeled biosynthetically with [$^3$H]palmitic acid (34), such labeling is not conclusive evidence that the lipid is palmitic acid. p60$^{src}$, the transforming tyrosine protein kinase of Rous sarcoma virus, contains exclusively myristic acid, yet it can be labeled with [$^3$H]palmitic acid as a result of metabolism of the radioactive precursor (3). Because the acylation of p21$^{v\text{-}H\text{-}ras}$ appears to be important for transformation, we identified directly the lipid and examined the various forms of the protein to which it is attached.

## MATERIALS AND METHODS

**Cells and viruses.** The HA 8-21 line of murine NIH/3T3 cells transformed by HaSV (Ha-3T3 cells [21]) and BALB/3T3/A31 cells transformed by Kirsten sarcoma virus (1) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum. The same medium was used for growth of human A431 cells infected with HaSV (6) and the HBL-100 line of normal human mammary cells (F. P. Polanowski and E. V. Gaffney, In Vitro **12**:328, 1976).

**Biosynthetic labeling.** 9,10-[$^3$H]palmitic acid (23.5 Ci/mmol) or 9,10-[$^3$H]myristic acid (12.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was dried, dissolved in dimethyl sulfoxide as described previously (34), and diluted to 1 mCi/ml with DMEM and 10% calf serum. Cells were labeled with [$^{35}$S]methionine (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or [$^{35}$S]cysteine (>1,000 Ci/mmol; Amersham) in methionine- or cysteine-free DMEM containing 10% dialyzed calf serum for periods of up to 3 h or, for overnight labeling, in the same medium supplemented with 10% complete DMEM. Cells were labeled with 1 mCi of $^{32}P_i$ (carrier-free; ICN Pharmaceuticals Inc., Irvine, Calif.) per ml in phosphate-free DMEM with 10% dialyzed calf serum.

**Immunoprecipitation and polyacrylamide gel electrophoresis.** Lysis of cells in RIPA buffer and immunoprecipitation with monoclonal antibody Y13-259 and goat anti-rat-immunoglobulin G serum (13) were carried out as described previously (32). This antibody cross-reacts with both viral p21$^{v\text{-}H\text{-}ras}$ and p21$^{v\text{-}K\text{-}ras}$ as well as with cellular p21$^{c\text{-}ras}$ (13). p21$^{v\text{-}H\text{-}ras}$ and p21$^{c\text{-}ras}$ in human cells were immunoprecipitated with a rat antitumor serum which recognizes both viral and cellular proteins (32) (see Fig. 2). To decrease the background of nonspecifically bound proteins, fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring, La Jolla, Calif.) was boiled in 3% sodium dodecyl sulfate (SDS)–10% 2-mercaptoethanol (26) prior to use. Samples were analyzed on 17.5 or 20% polyacrylamide gels (33). Labeled proteins were detected by fluorography with presensitized film (2).

**Analysis of the fatty acid in [$^3$H]palmitic acid-labeled p21$^{v\text{-}H\text{-}ras}$.** p21$^{v\text{-}H\text{-}ras}$ and pp21$^{v\text{-}H\text{-}ras}$ were isolated by immunoprecipitation from $10^6$ Ha-3T3 cells labeled overnight with

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5 mCi of [$^3$H]palmitic acid in 5 ml of medium. Protein was purified by preparative polyacrylamide gel electrophoresis, eluted from the gel (34), and hydrolyzed for 18 h at 110°C in 6 N HCl. The released fatty acids were extracted into toluene and analyzed by ascending chromatography on $KC_{18}$ reversed phase plates (Whatman, Inc., Clifton, N.J.) in acetic acid-acetonitrile (1:1) (3). The developed plate was dried, dipped in 2-methylnapthalene containing 0.4% diphenyloxazole, and exposed to presensitized film at −70°C.

**Partial proteolytic mapping.** p21$^{v\text{-}H\text{-}ras}$ and pp21$^{v\text{-}H\text{-}ras}$ were obtained from gels that had not been prepared for fluorography. The position of [$^3$H]palmitic acid-labeled p21$^{v\text{-}H\text{-}ras}$ was interpolated from that of adjoining [$^{35}$S]methionine-labeled p21$^{v\text{-}H\text{-}ras}$ markers. Partial proteolysis of the proteins was performed on 24% polyacrylamide gels with 250 ng of *S. aureus* V-8 protease, as described previously (5, 34).

## RESULTS

**p21$^{v\text{-}H\text{-}ras}$ contains palmitic acid.** To determine the identity of the fatty acid linked to p21$^{v\text{-}H\text{-}ras}$, cultures of Ha-3T3 cells (21) were labeled for 2 h with either 1 mCi of [$^3$H]palmitic acid or 1 mCi of [$^3$H]myristic acid, and p21$^{v\text{-}H\text{-}ras}$ was isolated by immunoprecipitation. While p21$^{v\text{-}H\text{-}ras}$ from [$^3$H]palmitic acid-labeled cells was easily detected, very little labeling of p21$^{v\text{-}H\text{-}ras}$ with [$^3$H]myristic acid was observed (Fig. 1A). [$^3$H]myristic acid, however, was incorporated into other proteins in these cells (data not shown). This suggests that the lipid in p21$^{ras}$ is more likely to be palmitic acid than myristic acid. To identify the lipid directly, p21$^{v\text{-}H\text{-}ras}$ was purified from [$^3$H]palmitic acid-labeled cells by immunoprecipitation and polyacrylamide gel electrophoresis. The two acylated forms, p21$^{v\text{-}H\text{-}ras}$ and pp21$^{v\text{-}H\text{-}ras}$ (34), were hydrolyzed with 6 N HCl, and the released lipid was analyzed by thin-layer chromatography on reversed phase plates. Palmitic acid and a small amount of stearic acid were detected (Fig. 1B). No myristic acid was found.

**A subpopulation of p21$^{v\text{-}H\text{-}ras}$ molecules is acylated.** p21$^{v\text{-}H\text{-}ras}$ undergoes extensive posttranslational processing, which yields a family of proteins which can be resolved into as many as five species by SDS-polyacrylamide gel electrophoresis (34) (Fig. 2). The mature forms p21$^{v\text{-}H\text{-}ras}$ and phosphorylated pp21$^{v\text{-}H\text{-}ras}$ contain lipid, while the precursor *pro*-p21$^{v\text{-}H\text{-}ras}$ does not (34). However, both palmitic acid-labeled p21$^{v\text{-}H\text{-}ras}$ and palmitic acid-labeled pp21$^{v\text{-}H\text{-}ras}$ migrated slightly faster than the majority of their respective forms (Fig. 2). The [$^3$H]palmitic acid-labeled proteins were clearly authentic p21$^{ras}$ from the pattern of fragments generated by partial proteolysis (see below). Densitometry revealed that less than 15% of the nonphosphorylated, mature forms of p21$^{v\text{-}H\text{-}ras}$ protein migrated in the position of the smaller palmitylated species. Apparently only a subpopulation of the mature forms of p21$^{v\text{-}H\text{-}ras}$ was acylated.

The smaller [$^3$H]palmitic acid-labeled band had a mobility somewhat similar to p21$^{c\text{-}ras}$, and the Y13-259 monoclonal antibody used here recognized p21$^{c\text{-}ras}$. We therefore examined whether this smaller acylated species was p21$^{c\text{-}ras}$. p21$^{c\text{-}ras}$ from [$^{35}$S]methionine-labeled NIH/3T3 cells was 13% as abundant as the viral p21$^{v\text{-}H\text{-}ras}$ protein in Ha-3T3 cells (Fig. 2). We could detect no palmitylated p21$^{c\text{-}ras}$ in the NIH/3T3 cells with an exposure time in which the [$^3$H]palmitic acid-labeled protein in Ha-3T3 cells was easily visible. There was not enough palmitylated p21$^{c\text{-}ras}$ to account for the amount of the smaller form of [$^3$H]palmitic acid-labeled protein in the Ha-3T3 cells.

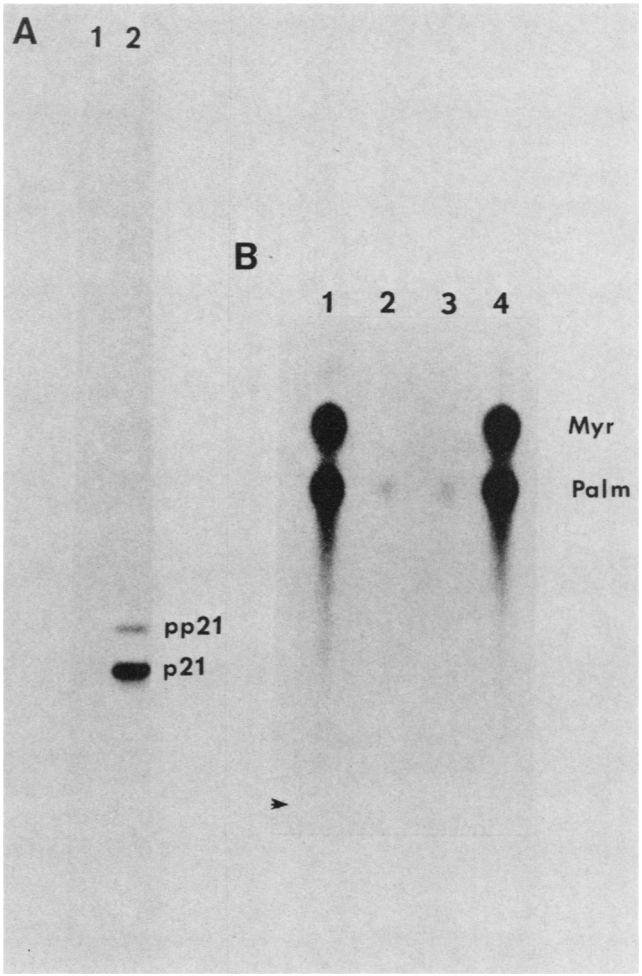


FIG. 1. (A) Comparison of the incorporation of [$^3$H]myristic acid and [$^3$H]palmitic acid into p21$^{v\text{-}H\text{-}ras}$. Ha-3T3 cells ($10^6$) were labeled for 2 h with either 1 mCi of [$^3$H]myristic acid or 1 mCi of [$^3$H]palmitic acid. p21$^{v\text{-}H\text{-}ras}$ was isolated by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis. p21$^{v\text{-}H\text{-}ras}$ was detected by fluorography after an exposure time of 22 days. Lane 1, [$^3$H]myristic acid-labeled cells; lane 2, [$^3$H]palmitic acid-labeled cells. (B) Analysis of radioactive fatty acids in p21$^{v\text{-}H\text{-}ras}$ labeled biosynthetically with [$^3$H]palmitic acid. The bands corresponding to [$^3$H]palmitic acid-labeled pp21$^{v\text{-}H\text{-}ras}$ and p21$^{v\text{-}H\text{-}ras}$ were cut from a preparative gel similar to that described for panel A. Protein was eluted and hydrolyzed with 6 N HCl overnight at 110°C. One-half of the released fatty acids were analyzed by ascending chromatography on a reversed phase thin-layer plate. The origin is indicated by an arrow. The plate was exposed to film for 60 days. Lanes 1 and 4, Mixture of [$^3$H]myristic acid (Myr) and [$^3$H]palmitic acid (Palm) standards; lane 2, fatty acid released from p21$^{v\text{-}H\text{-}ras}$; lane 3, fatty acid released from pp21$^{v\text{-}H\text{-}ras}$.

A cellular p21$^{c\text{-}ras}$ was also observed in the HBL-100 line of normal human mammary cells labeled with [$^{35}$S]methionine (Fig. 2). This p21$^{c\text{-}ras}$ protein could be labeled detectably with [$^3$H]palmitic acid. Palmitylation was thus not a unique modification of viral or mutated cellular forms of p21$^{ras}$. The palmitylated form of human cellular p21$^{c\text{-}ras}$ expressed in these cells migrated more rapidly than the viral p21$^{v\text{-}H\text{-}ras}$ on polyacrylamide gels. Thus in both human and NIH/3T3 cells transformed by HaSV, the smaller [$^3$H]palmitic acid-labeled protein was a viral rather than a cellular p21$^{ras}$ protein.

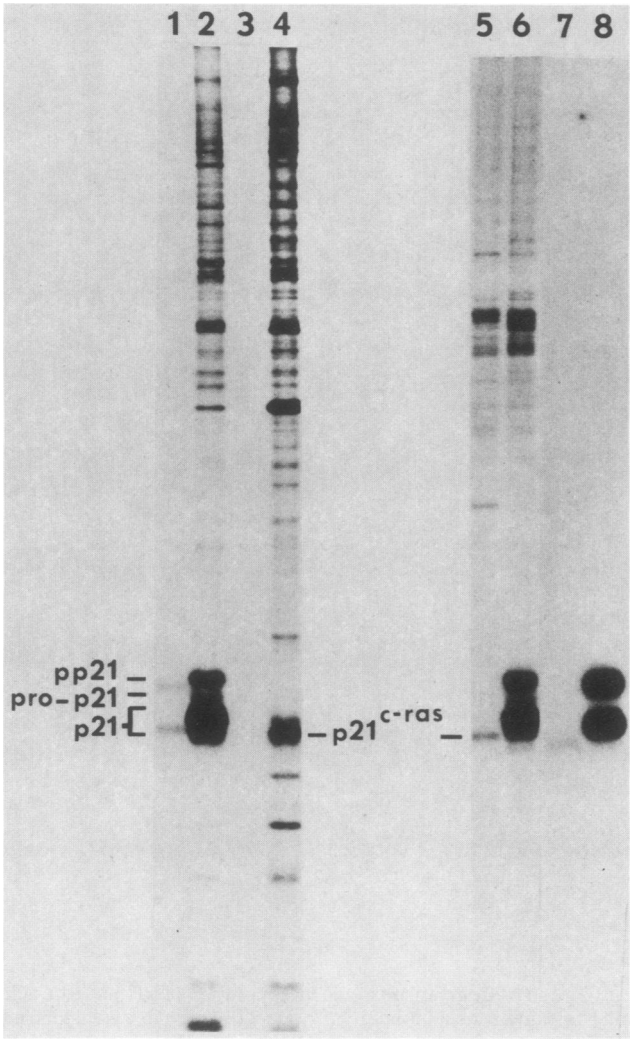


FIG. 2. Comparison of the forms of $p21^{v\text{-}H\text{-}ras}$ and $p21^{c\text{-}ras}$ labeled with [$^{35}$S]methionine or [$^{3}$H]palmitic acid. Lanes 1 through 4, Uninfected NIH/3T3 or Ha-3T3 cells were labeled overnight with 100 μCi of [$^{35}$S]methionine per ml or with 2 mCi of [$^{3}$H]palmitic acid per ml. $p21^{v\text{-}H\text{-}ras}$ and $p21^{c\text{-}ras}$ were isolated from equal numbers of cells ($10^6$) by immunoprecipitation with monoclonal antibody Y13-259 and analyzed by electrophoresis on a 17.5% polyacrylamide gel. Fluorographic exposure was for 21 days. Lanes 5 through 8, The HBL line of normal human mammary cells were labeled for 4 h with 1 mCi of [$^{3}$H]palmitic acid per ml or 100 μCi of [$^{35}$S]methionine per ml. Human A431 cells infected with HaSV (Ha-A431) were labeled for 4 h with 100 μCi of [$^{35}$S]methionine per ml or overnight with 1 mCi of [$^{3}$H]palmitic acid per ml. $p21^{v\text{-}H\text{-}ras}$ and $p21^{c\text{-}ras}$ were isolated by immunoprecipitation with a rat antitumor serum and analyzed by electrophoresis on a 20% polyacrylamide gel. Fluorographic exposure was for approximately 30 days. Lane 1, [$^{3}$H]palmitic acid-labeled Ha-3T3 cells; lane 2, [$^{35}$S]methionine-labeled Ha-3T3 cells; lane 3, [$^{3}$H]palmitic acid-labeled 3T3 cells; lane 4, [$^{35}$S]methionine-labeled 3T3 cells; lane 5, [$^{35}$S]methionine-labeled HBL cells; lane 6, [$^{35}$S]methionine-labeled Ha-A431 cells; lane 7, [$^{3}$H]palmitic acid-labeled HBL cells; lane 8, [$^{3}$H]palmitic acid-labeled Ha-A431 cells.

**Kirsten $p21^{ras}$ is acylated.** Two $p21^{v\text{-}K\text{-}ras}$ proteins with apparent molecular masses of 22 and 19 kilodaltons (kDa) were detected in Kirsten sarcoma virus-transformed mouse cells labeled with [$^{35}$S]methionine for 10 min (Fig. 3). The relative amount of the smaller, 19-kDa protein increased as the labeling time was lengthened, suggesting that it is a processed form of the 22-kDa species. The 22-kDa form was detected irrespective of the labeling period and persisted during a 2-h chase (data not shown). After labeling times of greater than 3 h, two additional forms of $p21^{v\text{-}K\text{-}ras}$ were detected: one with an apparent molecular mass of 22.5 kDa and a fourth form of approximately 24 kDa. In samples labeled for 18 h, the 19-kDa form could also occasionally be resolved into two species. The 24-kDa species was the only form which was labeled with $^{32}P_i$ (Fig. 3) and thus was the phosphorylated form of $p21^{v\text{-}K\text{-}ras}$. Each of these forms of $p21^{v\text{-}K\text{-}ras}$ could be seen in cells labeled for 18 h. [$^{3}$H]palmitic

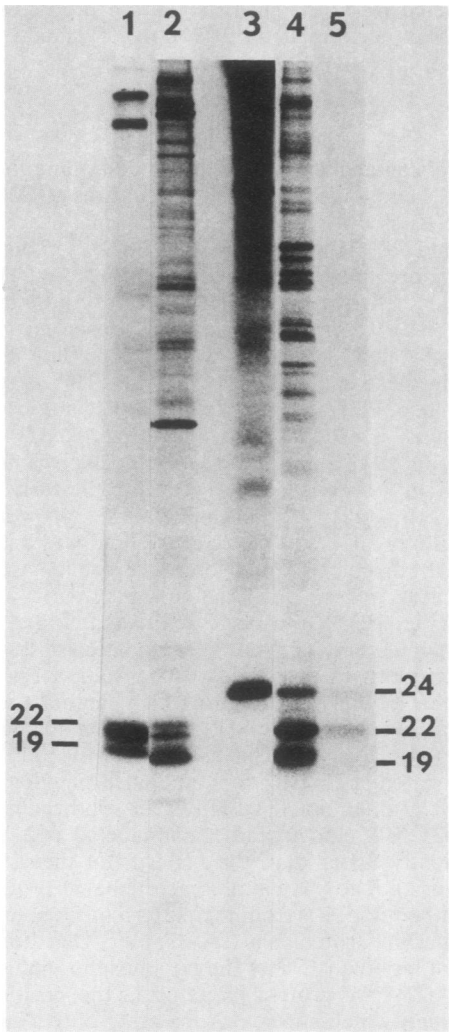


FIG. 3. Three forms of Kirsten sarcoma virus $p21^{v\text{-}K\text{-}ras}$ are acylated. Kirsten sarcoma virus-transformed NIH/3T3 cells were labeled for 10 min with 200 μCi, for 3.5 h with 100 μCi, or for 18 h with 50 μCi of [$^{35}$S]methionine per ml. Two additional cultures were labeled with 1 mCi of [$^{3}$H]palmitic acid per ml or 1 mCi of $^{32}P_i$ per ml for 18 h. $p21^{v\text{-}K\text{-}ras}$ was isolated by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis. The lanes containing samples labeled with $^{32}P_i$ or with [$^{35}$S]methionine for 3.5 h were exposed to film for 1 day. Fluorographic exposure of the remaining lanes was for 35 days. Lane 1, Cells labeled for 10 min with [$^{35}$S]methionine; lane 2, 3.5-h label with [$^{35}$S]methionine; lane 3, 18-h label with $^{32}P_i$; lane 4, 18-h label with [$^{35}$S]methionine; lane 5, 18-h label with [$^{3}$H]palmitic acid. Numbers to the right and left of the gels are in kilodaltons.

acid was incorporated into three p21$^{ras}$ proteins in the Kirsten sarcoma virus-transformed cells (Fig. 3). One acylated protein corresponded to the 24-kDa pp21$^{v\text{-}K\text{-}ras}$. Both the 19- and 22-kDa forms of p21$^{v\text{-}K\text{-}ras}$ contained [$^3$H]palmitic acid. This was unexpected, because the 22-kDa precursor form of p21$^{v\text{-}H\text{-}ras}$ does not contain palmitate (Fig. 2). With the two larger proteins (24 and 22 kDa), the [$^3$H]palmitic acid- and [$^{35}$S]methionine-labeled proteins comigrated. The acylated form of the 19-kDa protein, however, migrated slightly faster than the [$^{35}$S]methionine-labeled protein.

**Partial proteolysis of p21$^{v\text{-}H\text{-}ras}$.** Where is the palmitate located on p21$^{v\text{-}H\text{-}ras}$? Willumsen and co-workers have suggested that cysteine 186 is the site of palmitate attachment to HaSV p21$^{v\text{-}H\text{-}ras}$ (47). Our results from partial proteolysis of the intact HaSV p21$^{v\text{-}H\text{-}ras}$ with *S. aureus* V-8 protease are consistent with a carboxy-terminal location for all of the lipid in p21$^{v\text{-}H\text{-}ras}$. Digestion of [$^{35}$S]cysteine-labeled p21$^{v\text{-}H\text{-}ras}$ produced three labeled fragments, with sizes of 15, 14, and 7 kDa (Fig. 4). Digestion of p21$^{v\text{-}H\text{-}ras}$ labeled with [$^{35}$S]methionine showed that the 15- and 14-kDa peptides contained methionine but that the 7-kDa peptide did not. Treatment of p21$^{v\text{-}H\text{-}ras}$ with as much as 500 ng of V-8 protease did not alter this pattern of fragmentation, indicating that both the 14- and 15-kDa peptides represent end products (data not shown). Proteolysis of [$^3$H]palmitic acid-labeled p21$^{v\text{-}H\text{-}ras}$ demonstrated that both the 14- and 15-kDa fragments contain lipid. No [$^3$H]palmitic acid label could be detected in the 7-kDa peptide. Digestion of the phosphorylated form pp21$^{v\text{-}H\text{-}ras}$ labeled with [$^{35}$S]methionine yielded one labeled fragment identical to the 14-kDa peptide seen with p21$^{v\text{-}H\text{-}ras}$ and a novel labeled fragment of 17 kDa. No 15-kDa fragment was detected. Proteolysis of [$^{35}$S]cysteine-labeled pp21$^{v\text{-}H\text{-}ras}$ produced labeled fragments of 14, 17, and 9 kDa. No 7-kDa peptide was apparent. Because the phosphorylation of p21$^{v\text{-}H\text{-}ras}$ alters its apparent molecular weight, proteolytic fragments which contain phosphate might also be expected to have an altered mobility. Digestion of $^{32}$P-labeled pp21$^{v\text{-}H\text{-}ras}$ confirmed that the 17- and 9-kDa fragments, which are unique to pp21$^{v\text{-}H\text{-}ras}$, contained phosphate, while the 14-kDa peptide did not. Palmitate was found in both the 17- and 14-kDa fragments but not in the 9-kDa fragment of pp21$^{v\text{-}H\text{-}ras}$.

## DISCUSSION

The results presented here demonstrate directly that the fatty acid attached to HaSV p21$^{v\text{-}H\text{-}ras}$ is palmitic acid. No myristic acid was found. While it is clear that palmitic acid and a small amount of stearic acid are the only fatty acids attached to p21$^{v\text{-}H\text{-}ras}$, the exact linkage between acyl group and polypeptide remains to be determined. We have shown previously that the bond between p21$^{v\text{-}H\text{-}ras}$ and fatty acid is likely to be an ester or thioester, based on its susceptibility to cleavage by hydroxylamine (34). The palmitic acid could be attached directly to an amino acid, probably a cysteine residue (see below) or, alternatively, through an intervening moiety such as ethanolamine or glycerol similar to the variant surface glycoprotein of trypanosomes (12) or bacterial lipoprotein (15). Chen et al. (4) have shown that palmitic acid is the only lipid present in the carboxy-terminal tryptic peptide of p21$^{v\text{-}H\text{-}ras}$.

Kirsten sarcoma virus p21$^{v\text{-}K\text{-}ras}$ also contains lipid. All three of the major forms of p21$^{v\text{-}K\text{-}ras}$ are acylated. It is possible that the smallest, palmitic acid-labeled p21$^{ras}$ protein in the Kirsten sarcoma virus-transformed cells is p21$^{c\text{-}ras}$, because the apparent molecular weights of the cellular protein and viral proteins are very similar. However, the very small amount of [$^3$H]palmitic acid-labeled p21$^{c\text{-}ras}$ protein in NIH/3T3 cells suggests strongly that the much more abundant species in the virally transformed cells is p21$^{v\text{-}K\text{-}ras}$, not p21$^{c\text{-}ras}$.

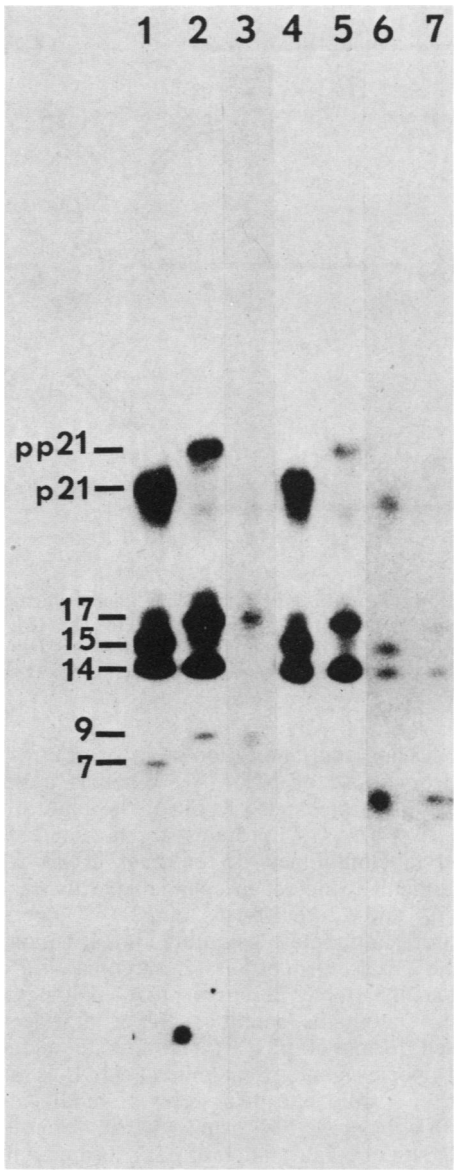


FIG. 4. Partial proteolysis of p21$^{ras}$ and pp21$^{ras}$. Ha-3T3 cells were labeled for 18 h with 100 μCi of [$^{35}$S]cysteine or [$^{35}$S]methionine per ml or 1 mCi of [$^3$H]palmitic acid per ml. An additional culture was labeled for 3 h with 1 mCi of $^{32}P_i$ per ml. p21$^{ras}$ proteins were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. Gel slices that contained p21$^{ras}$ or pp21$^{ras}$ were then subjected to partial proteolytic digestion with 250 ng of *S. aureus* protease and analyzed on a 24% polyacrylamide gel. The digestion products were detected by fluorography for 30 days for [$^{35}$S]cysteine- and [$^{35}$S]methionine-labeled proteins, for 60 days for [$^3$H]palmitic acid-labeled protein, and for 12 days with an intensifying screen for $^{32}$P-labeled protein. The film was blemished in the 60-day exposure for [$^3$H]palmitic acid-labeled proteins. The 30-day exposure showed no labeled peptides in this region. Lane 1, [$^{35}$S]cysteine-labeled p21$^{ras}$; lane 2, [$^{35}$S]cysteine-labeled pp21$^{ras}$; lane 3, $^{32}$P-labeled pp21$^{ras}$; lane 4, [$^{35}$S]methionine-labeled p21$^{ras}$; lane 5, [$^{35}$S]methionine-labeled pp21$^{ras}$; lane 6, [$^3$H]palmitic acid-labeled p21$^{ras}$; lane 7, [$^3$H]palmitic acid-labeled pp21$^{ras}$.

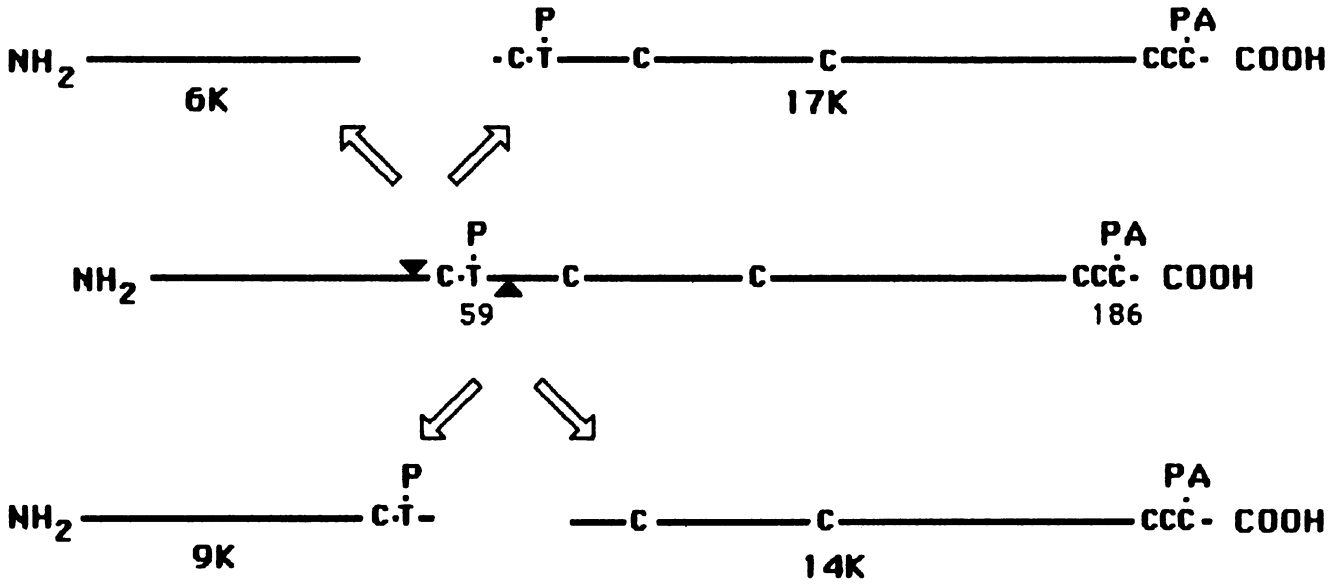


FIG. 5. Representation of [$^{35}$S]cysteine-labeled fragments derived from p21$^{v\text{-}H\text{-}ras}$ by partial proteolysis with *S. aureus* V8 protease. The six cysteine (C) residues at positions 51, 80, 118, 181, 184, and 186 (9) are indicated. T · P represents the phosphorylated threonine at position 59, and PA symbolizes the palmitic acid. Numbers indicate the apparent molecular weights (in thousands [K]) of the peptide fragments generated by alternative cleavage at two sites (arrows) which flank threonine 59.

The processing and maturation steps of p21$^{v\text{-}K\text{-}ras}$ appear to differ from those of p21$^{v\text{-}H\text{-}ras}$. Whereas a single *pro*-p21$^{v\text{-}Ha\text{-}ras}$ protein gives rise to all of the mature forms of p21$^{v\text{-}H\text{-}ras}$, two p21$^{v\text{-}K\text{-}ras}$ proteins are detected even with very short labeling times. In contrast to *pro*-p21$^{v\text{-}Ha\text{-}ras}$, which is difficult to detect in cells labeled to steady state, both the 19- and 22-kDa forms of p21$^{v\text{-}K\text{-}ras}$ persist. *pro*-p21$^{v\text{-}H\text{-}ras}$ does not contain palmitate (34). It is not yet clear whether the 22-kDa form of Kirsten sarcoma virus p21$^{v\text{-}K\text{-}ras}$ is an unusually stable percursor that, unlike the HaSV *pro*-p21$^{v\text{-}H\text{-}ras}$, contains palmitic acid or if processed and unprocessed forms of p21$^{v\text{-}K\text{-}ras}$ comigrate and only the processed species contains palmitic acid. It is likely that palmitylation is only one of a series of modifications that occurs with either p21$^{v\text{-}H\text{-}ras}$ or p21$^{v\text{-}K\text{-}ras}$.

The only site of palmitylation of p21$^{ras}$ is apparently in the carboxyl region of the protein (Fig. 5). Digestion of [$^{35}$S]cysteine-labeled phosphorylated pp21$^{v\text{-}H\text{-}ras}$ with *S. aureus* V-8 protease yields equal amounts of labeled 14- and 17-kDa peptides and a labeled fragment of 9 kDa. The 17- and 9-kDa fragments both contain the site of phosphorylation, theonine 59 (35). The 14-kDa fragment does not. The 14- and 17-kDa fragments are too large to represent two halves of p21$^{v\text{-}H\text{-}ras}$. Since the fragments must overlap, they most probably arise from alternative cleavage of p21$^{v\text{-}H\text{-}ras}$ at two sites which flank threonine 59. Cleavage of pp21$^{v\text{-}H\text{-}ras}$ at one site would yield a 14-kDa fragment containing the carboxyl end and the phosphorylated 9-kDa fragment containing the amino terminus. The other cleavage would produce the phosphorylated 17-kDa fragment and a 6-kDa amino-terminal peptide that we did not detect because it lacks both phosphate and cysteine. All of the cysteine residues in p21$^{v\text{-}H\text{-}ras}$ are in the C-terminal two-thirds of the molecule (9). Both the 14- and 17-kDa fragments contain lipid. This places the site of palmitylation in the carboxyl domain of p21$^{v\text{-}H\text{-}ras}$. In addition, there appear to be no sites for lipid attachment in the amino-terminal portion of pp21$^{v\text{-}H\text{-}ras}$, since no [$^{3}$H]palmitic acid was detected in the 6- or 9-kDa fragments. These data extend the observations of Willumsen et al. (47) and Chen et al. (4) by demonstrating that no lipid is found outside the carboxy-terminal domain. The lack of N-terminal fatty acid in p21$^{v\text{-}H\text{-}ras}$ is significant, for the myristic acid found in p60$^{src}$ is attached to the N-terminal glycine residue. The position at which the fatty acid is attached is thus clearly different for these two acylated viral-transforming proteins.

All of the mature form(s) of p21$^{v\text{-}H\text{-}ras}$ are reported to be associated with membranes (36). Acylation of p21$^{v\text{-}H\text{-}ras}$ occurs posttranslationally, concurrent with the maturation of soluble *pro*-p21$^{v\text{-}H\text{-}ras}$ to the membrane-bound form, p21$^{v\text{-}H\text{-}ras}$ (34). We therefore suggested that lipid plays a role in the association of mature p21$^{v\text{-}H\text{-}ras}$ and pp21$^{v\text{-}H\text{-}ras}$ with membranes. While this may, indeed, be true, it appears that much of the population of mature p21$^{v\text{-}H\text{-}ras}$ may not contain lipid. The acylated forms of p21$^{v\text{-}H\text{-}ras}$ do not exactly comigrate with the major species of [$^{35}$S]methionine-labeled p21$^{v\text{-}H\text{-}ras}$ during polyacrylamide gel electrophoresis. Nevertheless, partial proteolysis proves that the [$^{3}$H]palmitic acid-labeled proteins are indeed p21$^{v\text{-}H\text{-}ras}$. While we can find no obvious reason for this paradoxical result, it is most readily explained by the lipid being present on only a minor population of p21$^{v\text{-}H\text{-}ras}$ molecules. This implies that successful interaction of mature p21$^{v\text{-}H\text{-}ras}$ with membranes must also occur without acylation and that other heretofore undetected modifications important for membrane binding may take place during conversion of *pro*-p21$^{v\text{-}H\text{-}ras}$ to mature p21$^{v\text{-}H\text{-}ras}$.

Two types of lipid modification of viral-transforming proteins can be discerned. Myristic acid is linked by an amide bond to the amino-terminal glycine residue of p60$^{src}$ (30) and several retroviral *gag* and *gag-onc* fusion proteins (16, 28, 29). The myristyl group is added to these proteins during or immediately after synthesis (2). Acylation of p21$^{v\text{-}H\text{-}ras}$ is different. The fatty acid present in p21$^{v\text{-}H\text{-}ras}$ is almost exclusively palmitic acid and is attached to p21$^{v\text{-}H\text{-}ras}$ posttranslationally, likely through an ester or thioester bond

(34). These are the same characteristics displayed by the palmitic acid present in the G glycoprotein of vesicular stomatitis virus and the HLA glycoprotein, in which palmitic acid is attached through a thioester bond to a cysteine residue (18, 27). Willumsen et al. (46) have shown that cysteine 186 of p21$^{v\text{-}H\text{-}ras}$ is essential for fatty acylation. Our data from the partial proteolysis of p21$^{v\text{-}H\text{-}ras}$ are consistent with a C-terminal location for the palmitic acid. An attractive hypothesis is that this cysteine is essential for palmitylation because it is the residue to which the palmitic acid is attached. Alternatively, cysteine 186 may be essential for the interaction of p21$^{v\text{-}H\text{-}ras}$ with cellular membranes, and without this interaction palmitylation cannot occur. Nonpalmitylated p21$^{v\text{-}H\text{-}ras}$ proteins lack transforming activity (46, 47). This suggests that palmitylation is essential for transformation. If this is so, the subpopulation of p21$^{v\text{-}H\text{-}ras}$ molecules which are acylated may be more stably bound to membranes or interact more effectively with critical substrates than nonacylated molecules and may thus be more able to induce transformation. All p21$^{ras}$ proteins that have been studied contain palmitic acid. This list now includes Kirsten sarcoma virus p21$^{v\text{-}H\text{-}ras}$ and a human cellular p21$^{c\text{-}ras}$ (this study) and the 23-kDa *ras* protein of *Dictyostelium discoideum* (25; T. Pawson, personal communication). The acylation of *ras* proteins from such different sources argues that this event is important for p21$^{ras}$.

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