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11. The CHO cells labeled with [5-^{3}H]mevalonate as described (*10*) were extracted with organic solvents to remove lipids. Cells (90 mg of total protein) were dried and sealed in an ampule with 0.2 ml of anhydrous hydrazine. After heating at 80°C for 24 hours, the hydrazine was removed under vacuum. The residue was tritrated with 2 ml of 50% 1-propanol-water and applied to a 1 cm by 3 cm column of Amberlite CG-50. Elution with 10 ml of the above solvent gave a 90% recovery of radioisotope. A small quantity of Raney nickel (W1) was added, and the mixture was stirred for 15 min. The hydrocarbons thus generated were extracted into pentane, which was washed with water and dried over Na_2SO_4. Chromatography on a 1 cm by 95 cm column of Bio-Beads S-X4 (Bio-Rad) using benzene as a solvent, and collecting 0.5-ml fractions gave the smaller hydrocarbon in fractions 51 to 55. The solvent was removed under a stream of N_2, and immediately prior to GC-MS analysis the sample was chromatographed on a micro column of silica gel with benzene as solvent. This sample contained 40% of the radioactivity based on the hydrazine product.
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16. Electron ionization mass spectra were acquired on a VG 70-SEQ instrument (VG Analytical, Manchester, United Kingdom): ionizing energy, 70 eV; accelerating potential, 8 kV; ion source temperature, 220°C. A Hewlett-Packard 5890 gas chromatograph interfaced directly to the mass spectrometer was used for sample introduction. Chromatography was performed on a fused silica DB-1 capillary column (15 m by 0.32 mm inside diameter with a 1.0-μm film thickness) (J and W Scientific, Inc., Folsom, California). The injector temperature was 280°C; the column was initially set at 80°C for (splitless) sample injection and held for 1 min, then programmed to 180°C at a rate of 25°C/min, and finally programmed to 290°C at a rate of 5°C/min.
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Identification of Geranylgeranyl-Modified Proteins in HeLa Cells

CHRISTOPHER C. FARNSWORTH, MICHAEL H. GELB,* JOHN A. GLOMSET*

Previous studies have shown that animal cells contain isoprenoid-modified proteins and that one of these proteins, lamin B, contains a thioether-linked farnesyl group that is attached to cysteine. In the present study, a novel isoprenoid-modification was identified by labeling HeLa cells with [^{3}H]mevalonic acid and analyzing proteolytic digests of the total cell protein. Radioactive fragments were purified from these digests and treated with Raney nickel. The released, labeled material was analyzed by gas-liquid chromatography (GC) and mass spectrometry (MS). This approach revealed that an all-*trans* geranylgeranyl group was a major isoprenoid modification.

STUDIES OF SWISS 3T3 CELLS THAT were labeled with [^{3}H]mevalonic acid provided the first evidence that animal cells contain proteins that are posttranslationally modified by isoprenoid groups. Several radioactive proteins were observed, and proteolytic hydrolysates of the total cell protein were shown to contain labeled fragments that had apparent molecular weights of 1000 and 500 daltons (*1*). This result suggested that different proteins in animal cells may be modified by different isoprenoid groups. Following these early observations, isoprenoid-modified proteins were detected in many other cell types (*2*). Furthermore, proteolytic fragments of HeLa cell proteins were prepared that resembled those derived from Swiss 3T3 cell proteins; one of the labeled proteins from HeLa cells was identified as lamin B (*3*) and was shown to contain a cysteinyl thioether-linked farnesyl group (*4*). Proteolytic digests of lamin B only yielded radioactive fragments that corresponded to the 500-dalton fragments of HeLa cell total proteins. Therefore, the identity of the isoprenoid modification in the 1000-dalton fragments was left unresolved.

To address this question we labeled HeLa cells with [^{3}H]mevalonic acid, extensively digested the total cell protein with proteases, and subfractioned the digests by anion exchange chromatography and gel filtration on Sephadex LH-20. Two peaks of radioactive material, termed peaks A and B, were obtained, and their contents were pooled into separate fractions (Fig. 1). The radioactive material was initially only slightly soluble in pentane, but became pentane-extractable following treatment of the fractions with Raney nickel (Table 1). In contrast, little or no additional radioactive material became pentane-soluble following treatment with methanolic KOH. This suggested that the radioactive material was linked to the protein fragments through thioether bonds.

The radioactive material that was released into pentane was analyzed by radiometric GC. A major peak of radioactive material derived from peak A eluted with a retention time of 44.0 min, which was identical to that of all-*trans* 2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene (compare A and C in Fig. 2). The major radioactive peak derived from peak B eluted significantly earlier, in the position of all-*trans* 2,6,10-trimethyl-2,6,10-dodecatriene (*4*). Parallel samples of the peak A–derived material that had been hydrogenated over platinum yield-

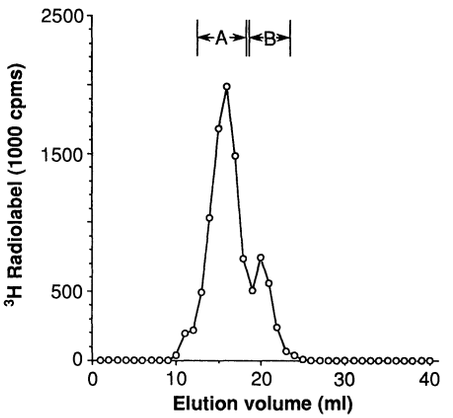


Fig. 1. Size-exclusion chromatography of proteolytic hydrolysates of HeLa cell total proteins on Sephadex LH-20. Cells were labeled for 36 hours with [5-^{3}H]mevalonic acid in the presence of 30 μ*M* mevinolin, harvested, washed with phosphate-buffered saline, and extracted with lipid solvents. Cell pellets were then successively digested with proteases, and labeled digestion products were concentrated and purified by step elution from DEAE Sephacel. The eluted material was then passed through Sephadex LH-20 in 20% formic acid in ethanol at a flow rate of 0.25 ml/min, and 1.0-ml fractions were collected (*4*). Peak A, which corresponded to 1000-dalton material, contained 74% of the recovered label. Peak B, which corresponded to 500-dalton material, contained 22% of the recovered label. Recovery of the total applied label was 78%. Comparable chromatograms were obtained in six separate experiments.

C. C. Farnsworth, Howard Hughes Medical Institute Laboratory, University of Washington, Seattle, WA 98195.
M. H. Gelb, Departments of Biochemistry and Chemistry, University of Washington, Seattle, WA 98195.
J. A. Glomset, Howard Hughes Medical Institute Laboratory, Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195.

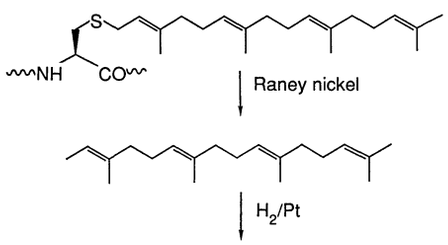
*To whom correspondence should be addressed.

Table 1. Release of ^3H label from proteolytic fragments of HeLa cell proteins. Material corresponding to peak A in Fig. 1 was solubilized in 200 μl of 8*M* guanidinium chloride, 50 mg of Raney nickel and 1.0 ml of pentane were added, and the samples were incubated for 15 hours at 100°C. Values are percentages of total peak A radioactivity. Control samples were treated similarly, but in the absence of Raney nickel. Additional peak A material was treated for 1 hour with 0.1*M* methanolic KOH at 23°C and then acidified and extracted with pentane (*4*). Values in parentheses represent numbers of experiments done.

Sample treatment	Relative radioactivity (percent)
+ Raney nickel (2)	88 ± 2
− Raney nickel (2)	14 ± 4
+ Methanolic KOH (3)	11 ± 0.5
− Methanolic KOH (3)	5 ± 0.1

ed a single peak of radioactivity that eluted in the position of phytane (2,6,10,14-tetramethylhexadecane) (compare B and D in Fig. 2).

The structure of the isoprenoid compound derived from peak A was established by combined GC-MS analysis. A parent ion peak with a mass-to-charge (*m/z*) value of 274 and a MS fragmentation pattern identical to that of all-*trans* 2,6,10,14-tetramethyl-2,6,10,14-hexadecatriene were observed (compare A and C in Fig. 3). Corresponding material from peak A that had been hydrogenated over platinum showed a parent ion peak with an *m/z* value of 282 (high-resolution MS analysis gave 282.3305; calculated for $C_{20}H_{42}$, 282.3325). The MS fragmentation pattern is identical to that of authentic phytane (compare B and D in Fig. 3). Raney nickel cleavage of the carbon-sulfur bond is predicted to generate 2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene (*3*), and further treatment with hydrogen and platinum would give phytane ($C_{20}H_{42}$) as shown below:



These results unequivocally demonstrate that the proteolytic fragments in peak A contained a covalently attached all-*trans* geranylgeranyl group and that geranylgeranyl groups can be attached to proteins of natural origin. The mode of attachment of this group to proteins remains to be established,

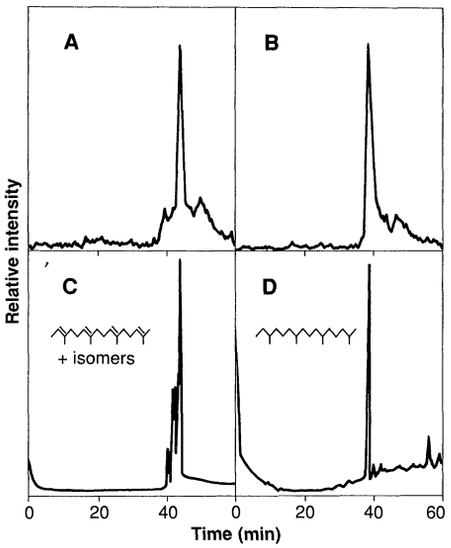


Fig. 2. Gas chromatographic (GC) analyses of labeled, Raney nickel–released material from proteolytic fragments of HeLa cell proteins. Tritium-labeled material corresponding to peak A (Fig. 1) was treated with Raney nickel and extracted with pentane as described in Table 1. (**A**) Pentane-soluble label was then concentrated, and one-half of it was analyzed. (**B**) The remainder was hydrogenated over platinum and analyzed. (**C**) A mixture of all eight possible cis-*trans* isomers of 2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene, which yielded eight distinct peaks when analyzed by capillary GC, was cochromatographed with the all-*trans* isomer (major peak). (**D**) Authentic phytane was also analyzed. Analyses were performed on a 15-m DB-5 megabore column with a temperature program that started at 80°C for 2 min, then increased 2°C per minute up to 220°C. Peak mass was detected by flame ionization, and radioactivity was detected with a Flow-One Beta Detector (Radiomatic Instrument Co., Tampa, Florida) (*4*). In this system the all-*trans* and nearest *cis*-containing tetraene isomers were separated sufficiently (0.4 min) to ensure unambiguous identification of the protein-derived material as the all-*trans* tetraene. The all-*trans* tetraene was synthesized from all-*trans* geranylgeraniol by conversion to the bromide followed by treatment with superhydride (*9*). The isomeric mixture of tetraenes was synthesized by a Wittig reaction between commercially available farnesyl acetone (mixture of all possible cis-trans isomers) and the ylide derived from (ethyl)triphenylphosphonium bromide (*4*). The structures of all of the synthetic compounds were verified by high-resolution proton nuclear magnetic resonance.

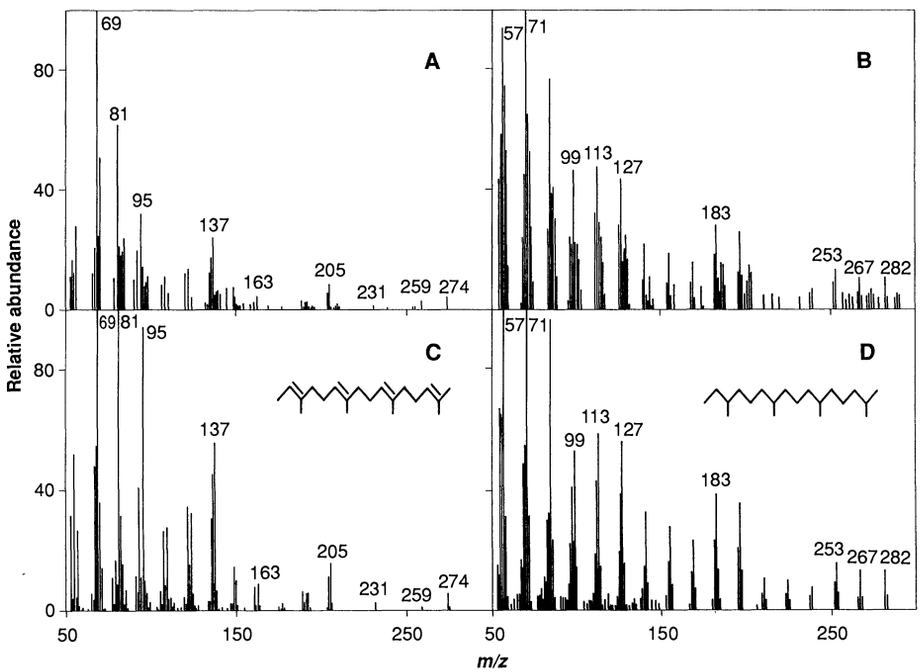


Fig. 3. Electron ionization spectra of Raney nickel–released material from proteolytic fragments of HeLa cell proteins. Samples were prepared from 3 liters of cultured cells as described in Figs. 1 and 2, then analyzed by GC-MS on a 30-m DB-5 column, before or after hydrogenation (*3*). (**A**) Nonhydrogenated, peak A–derived material corresponding to the major peak of radioactivity in Fig. 2A. (**B**) Hydrogenated, peak A–derived material corresponding to the peak of radioactivity in Fig. 2B. (**C**) All-*trans* 2,6,10,14-tetramethyl-2,6,10,14-hexadecatriene. (**D**) Phytane. All spectra have been background corrected, and the spectra in (A) and (C) have been enhanced. Minor differences between spectra of protein-derived samples and standards are likely due to the much smaller amounts of naturally derived materials analyzed.

but its release by treatment with Raney nickel strongly suggests that it is attached to cysteine through a thioether bond. Although the proteins that are modified with a geranylgeranyl group and the prenyltransferase that attaches the isoprenoid group to proteins remain to be identified, geranylgeranyl pyrophosphate is probably a substrate in the reaction. An enzyme that synthesizes this isoprenoid polyphosphate from isopen-

tenyl pyrophosphate and farnesyl pyrophosphate has been purified from pig liver (*5*).

It will be important to identify the geranylgeranyl-modified proteins and study their structures in view of current information regarding farnesylated proteins. The cDNA-derived amino acid sequences of those farnesylated proteins that have been identified contain a conserved CAAX motif at the COOH-terminus (where C is cysteine and A and X refer to aliphatic and any amino acid, respectively). Furthermore, prenylation occurs on the cysteine in this motif (*4, 6*), and ras proteins may be similarly modified (*6–8*).

Several of the farnesylated proteins also have been shown to be further modified. The last three amino acids (AAX) are removed from the COOH-terminus, and the newly exposed cysteine α-carboxyl group is converted to the methyl ester (*7, 8*). The CAAX motif may be the critical recognition element in this modification pathway because engineering of this sequence onto the COOH-terminus of a heterologous, cytosolic protein is sufficient to direct prenylation of the protein (*7*). In the cases so far examined, the isoprenoid group seems to be required for membrane localization and biological activity (*6–8*).

Similar possibilities warrant consideration in the case of geranylgeranyl-modified proteins. Therefore, a major task is to determine whether these proteins contain a directing structural motif such as the CAAX sequence, whether further posttranslational modifications occur, and whether these modifications play an important biological role.

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A Dominant Mutation That Predisposes to Multiple Intestinal Neoplasia in the Mouse

AMY RAPAICH MOSER,* HENRY C. PITOT, WILLIAM F. DOVE

In a pedigree derived from a mouse treated with the mutagen ethylnitrosourea, a mutation has been identified that predisposes to spontaneous intestinal cancer. The mutant gene was found to be dominantly expressed and fully penetrant. Affected mice developed multiple adenomas throughout the entire intestinal tract at an early age.

AN UNDERSTANDING OF NEOPLASIA is enhanced by the identification of genes that positively or negatively affect this process. Transgenic mice carrying activated oncogenes provide one animal model system that is proving to be useful (*1*). A complementary approach is to identify animals carrying single germline mutations that result in a susceptibility to tumor formation. The efficient production of mice carrying such mutations is possible by use of germline mutagenesis with ethylnitrosourea (Enu) combined with screening for a defined mutant phenotype. Ethylnitrosourea induces forward germline mutations at a frequency up to 1 in 700 per locus (*2*). Usually, these are single base pair changes (*3*). Either gain-of-function or loss-of-function mutations can be induced; this broadens the set of genes that can be detected by a mutational analysis.

We report a mutation in the mouse genome that leads to multiple intestinal tumors in all carriers. The mutation was identified in a pedigree established during a mutagenesis project in which C57BL/6J (B6) males were treated with Enu and then mated to AKR/J (AKR) females (*4*). One offspring from such a cross, an (AKR × B6)F_1 female that exhibited a circling behavior, was mated to a B6 male to test for the heritability of the circling trait. A progressive, adult-onset anemia was noted in some of the progeny. The anemia appeared to be transmitted as an autosomal dominant trait. The circling behavior was also shown to be heritable, but genetically unlinked to the anemia, and is no longer carried in this pedigree.

The hematological status of the animals was assessed by hematocrit determinations at regular intervals. Animals that showed a progressive decrease in hematocrit value were classified as anemic; those animals with normal hematocrit values (45 to 55%) were classified as unaffected. Blood counts revealed a progressive anemia characterized by a decreasing red cell count and an increasing proportion of reticulocytes, symptoms consistent with an anemia due to chronic blood loss. Moribund mice usually had hematocrit values in the range of 10 to 20%. This severe chronic anemia is thought to be the cause of lethality. In addition, nearly all anemic animals exhibited lipemia.

Anemic animals frequently passed bloody feces and had numerous visible tumors of the large and small intestine. The smallest visible tumors were about 1 mm in diameter, the largest up to 8 mm. Histological analysis revealed that these tumors were primarily polypoid, papillary, or sessile adenomas (Fig. 1). In older animals, locally invasive tumors were seen, but no visible metastatic tumors were observed at necropsy. Small areas of carcinoma in situ (*5*) were sometimes found in sections of tissues from anemic animals. By contrast, no significant abnormalities were seen in tissues of nonanemic animals from this pedigree. The primary phenotype of mice carrying this mutation appears to be the development of multiple adenomas which progress to adenocarcinomas of the intestine in older mice; the anemia is presumably secondary. Therefore, we named the mutant gene multiple intestinal neoplasia (*Min*).

To facilitate the propagation and study of mutant animals, we needed a method for the detection of *Min*/+ mice other than scoring for tumors at necropsy. All of the anemic mice examined had intestinal tumors. In addition, if a mouse was not anemic by 150 days of age, then no tumors were visible on necropsy at 300 days of age. Therefore, anemia was used as a provisional marker with which to recognize *Min* carriers for breeding. This classification was confirmed for each mouse in the *Min* breeding colony by examination of the intestines at autopsy.

As scored by anemia, *Min* is transmitted by affected mice to 50% of progeny with an unbiased sex distribution, as is characteristic of a fully penetrant autosomal dominant trait. As an explicit test of penetrance, 20 nonanemic males from the pedigree were

A. R. Moser, McArdle Laboratory for Cancer Research, University of Wisconsin–Madison, Madison, WI 53706.
H. C. Pitot, McArdle Laboratory for Cancer Research and Department of Pathology, University of Wisconsin–Madison, Madison, WI 53706.
W. F. Dove, McArdle Laboratory for Cancer Research and Laboratory of Genetics, University of Wisconsin–Madison, Madison, WI 53706.

*To whom correspondence should be addressed.