protein structure, possibly in the primary sequence. In addition, it suggested that these amphiphilic, acylated proteins are not anchored in the membrane by COOH-terminal regions, but rather are anchored by the NH_2-terminal portion of the molecule. This orientation is common in the prokaryotic lipoproteins (*9, 14*) but has not been described in phase-variant surface antigen systems.

Thus, we have identified antigenically distinct sets of lipid-modified surface proteins in *M. hyorhinis* that undergo high-frequency reciprocal phase variation as well as size variation in isogenic populations. These variations can occur concomitantly with or independently from other reversible phenotypic transitions, raising the possibility of a coordinately regulated system perhaps modulated by external stimuli (*1*). The degree of diversification in separate, antigenically distinct sets of lipoproteins recognized in this species by other MAbs (*9*) is not known, but phenotypic changes in the expression of another prototype lipoprotein, p120 (*9, 15*), have also been observed in subcloned lineages (*12*). Phase variation of these or several other membrane lipoproteins (*15*) could be important in providing antigenic and functional flexibility in populations of these organisms. Genetic mechanisms generating this diversity are not yet understood, but previous reports of lysogenic viruses in this species (*16*) as well as the demonstration of potentially transmissible elements resembling prokaryotic insertion sequences (*17*) provide an impetus to analyze possible genomic rearrangements associated with the observed phenotype changes. These studies may reveal common and perhaps novel mechanisms used by other mycoplasmas that are also known to express intraspecies diversity in several phenotypic traits (*18*).

REFERENCES AND NOTES

1. J. F. Miller, J. J. Mekalanos, S. Falkow, *Science* **243**, 916 (1989).
2. H. S. Seifert and M. So, *Microbiol. Rev.* **52**, 327 (1988); J. E. Donelson, in *Mobile DNA*, D. E. Berg and M. M. Howe, Eds. (American Society for Microbiology, Washington, DC, 1989), pp. 763–781; D. R. Soll, *ibid.*, pp. 791–798.
3. R. F. Ross, *Ann. N.Y. Acad. Sci.* **225**, 347 (1973).
4. P. Whittlestone, in *The Mycoplasmas*, J. G. Tully and R. F. Whitcomb, Eds. (Academic Press, New York, 1979), vol. 2, pp. 133–176.
5. R. J. Hay, M. L. Macy, T. R. Chen, *Nature* **339**, 487 (1989).
6. M. F. Barile, in *The Mycoplasmas*, J. C. Tully and R. F. Whitcomb, Eds. (Academic Press, New York, 1979), vol. 2, pp. 425–474.
7. R. A. Del Giudice, R. S. Gardella, H. E. Hopps, *Curr. Microbiol.* **4**, 75 (1980).
8. M. Goiš, F. Kuksa, J. Franz, D. Taylor-Robinson, *J. Med. Microbiol.* **7**, 105 (1974).
9. M. J. Boyer and K. S. Wise, *Infect. Immun.* **57**, 245 (1989).
10. J. R. Duncan and R. F. Ross, *Am. J. Vet. Res.* **34**, 363 (1978).
11. B. A. D. Stocker, *J. Hyg.* **47**, 398 (1949).
12. R. Rosengarten and K. S. Wise, unpublished data.
13. R. Hayashi, S. Moore, W. H. Stein, *J. Biol. Chem.* **248**, 2296 (1973).
14. H. C. Wu and M. Tokunaga, *Curr. Top. Microbiol. Immunol.* **125**, 127 (1986); J. P. Woods, S. M. Spinola, S. M. Strobel, J. G. Cannon, *Mol. Microbiol.* **3**, 43 (1989).
15. T. M. Bricker, M. J. Boyer, J. Keith, R. Watson-McKown, K. S. Wise, *Infect. Immun.* **56**, 295 (1988).
16. R. N. Gourlay, S. G. Wyld, M. E. Poulton, *Arch. Virol.* **77**, 81 (1983).
17. R. V. Ferrell, M. B. Heidari, K. S. Wise, M. A. McIntosh, *Mol. Microbiol.* **3**, 957 (1989).
18. A. Liss and R. A. Heiland, *Infect. Immun.* **41**, 1245 (1983); A. Valdivieso-Garcia and S. Rosendal, *Vet. Rec.* **110**, 470 (1982); B. J. Deeb and G. E. Kenny, *J. Bacteriol.* **93**, 1416 (1967); H. L. Watson, L. S. McDaniel, D. K. Blalock, M. T. Fallon, G. H. Cassell, *Infect. Immun.* **56**, 1358 (1988); H. Andersen, S. Birkelund, G. Christiansen, E. A. Freundt, *J. Gen. Microbiol.* **133**, 181 (1987); D. C. Krause, D. K. Leith, J. B. Baseman, *Infect. Immun.* **39**, 830 (1983).
19. J. G. Tully, in *Methods in Mycoplasmology*, S. Razin and J. G. Tully, Eds. (Academic Press, New York, 1983), vol. 1, pp. 173–177.
20. To generate and analyze subcloned mycoplasma lineages, we initiated 1-ml broth cultures from single colony isolates and expanded them to a titer of $\sim 2 \times 10^9$ colony-forming units (CFU) per milliliter (representing 31 total generations) in growth medium supplemented with 20% heat-inactivated horse serum [K. S. Wise and R. K. Watson, *Infect. Immun.* **41**, 1332 (1983)]. The filtrates of broth cultures passed through a 0.2-μm pore-size filter were plated at various dilutions on 1% noble agar (Difco, Detroit, MI) medium of the same composition. After 6 days, colonies were photographed with transmitted and oblique lighting to register their morphology and opacity. To monitor expression of antigens, we transferred imprints of colonies to nitrocellulose filters [H. Kotani and G. J. McGarrity, *J. Clin. Microbiol.* **23**, 783 (1986)] and immunostained with MAb as described (*9*). Well-separated colonies with selected phenotypes were picked from agar plates, expanded as described in 1-ml broth cultures, and the resulting population again analyzed by additional rounds of plating, characterization of phenotypes, and colony isolation.
21. Single mycoplasma colonies of a selected phenotype were expanded to $\sim 2 \times 10^9$ CFU in 1-ml broth cultures. Organisms were either harvested and analyzed by SDS-PAGE, electrophoretic transfer to nitrocellulose, and immunostaining (*9*), or alternatively, were either labeled in cysteine-depleted medium supplemented with [^{35}S]cysteine (975 Ci/mmol, 0.5 mCi/ml; DuPont, Biotechnology Systems) or in growth medium supplemented with [^{3}H]palmitate (30 Ci/mmol, 1 mCi/ml; DuPont). The labeled cultures were subjected to Triton X-114 phase-fractionation, after which the detergent-phase proteins were analyzed by SDS-PAGE and fluorography as described (*15*). Blots of labeled detergent-phase proteins were immunostained and autoradiographed to confirm the correspondence of epitope-bearing and metabolically labeled proteins (*12*).

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Prenylated Proteins: The Structure of the Isoprenoid Group

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The mevalonate-derived portion of a prenylated protein from Chinese hamster ovary cells has been established as diterpenoid (C_{20}). This group is linked to a carboxyl-terminal cysteine as a thioether. It was removed from the protein by hydrazinolysis followed by Raney nickel desulfurization, and the resulting hydrocarbon fraction was analyzed by gas chromatography–mass spectrometry.

PRENYLATION, A RECENTLY DISCOVERED posttranslational modification of proteins, has become a topic of substantial interest (*1*). Prenylation is one of a series of reactions involved in converting Ras proteins to their active form (*1, 2*). Ras oncogene products play a prominent role in neoplastic processes, and overexpression of normal Ras protein has been linked to malignant transformation (*3*). Covalent modification of proteins and peptides by mevalonate-derived polyprenyl groups has been demonstrated in a number of tissues and is apparently a general phenomenon (*4–7*). Current thought concerning the events surrounding prenylation has come from consideration of similarities in the structure, function, and processing of Ras proteins and the a factor, a yeast mating pheromone. Precursor proteins, pro-proteins that are synthesized in the cytosol, share a common carboxyl-terminal sequence Cys-AAX, where A and X refer to aliphatic and any amino acid, respectively. Processing involves the proteolytic removal of the terminal tripeptide, exposing cysteine as the new carboxyl-terminal amino acid and converting the free carboxyl to its methyl ester. At some point in the process the cysteine is prenylated by alkylation of its sulfur by a polyprenyl donor, presumably the diphosphate. The identity of only one prenyl group has been established unequivocally; the yeast isoprenoid is the C_{15} farnesyl group (*8, 9*).

We studied prenylation of proteins in Chinese hamster ovary (CHO) cells and

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found two different sizes of polyisoprenoid groups that were covalently attached to cellular protein (*10*). Both groups are linked as thioethers to a carboxyl-terminal cysteine. The smaller isoprenoid has been isolated as a hydrocarbon by a cleavage reaction, and we report that on the basis of mass spectrometric analysis that it has a diterpene (C_{20}) structure.

The CHO-K1 cells were grown on media containing [5-^{3}H]mevalonate to label the prenyl groups (*11*). Prenylated cysteine was cleaved from total cellular protein by hydrazinolysis (*12*), and the neutral amino acid fraction was isolated by ion exchange chromatography. Treatment of this fraction with Raney nickel (*13*) released the prenyl groups as hydrocarbons. The smaller hydrocarbon was purified by gel exclusion chromatography (*11*) followed by chromatography on a small column of silica gel prior to analysis by gas chromatography–mass spectrometry (GC-MS).

The GC-MS data (Fig. 1) show four species that contain an isoprene function, identified by formation of the isoprene ion (mass-to-charge ratio *m/z* of 69) (*14*). Mass spectra of the two major components, peak 1 (Fig. 1B) and peak 2 (whose spectrum is essentially identical to that of peak 1) indicate a molecular weight of 274 daltons, supporting the presence of two $C_{20}H_{34}$ hydrocarbons with four degrees of unsaturation. The ions of *m/z* 69, 137, and 205 clearly are associated with isoprene fragments (*14*). The occurrence of two hydrocarbons may be due to two C_{20} prenylated cysteines whose double-bond geometry differs, or it may result from Raney nickel hydrogenolysis of one allylic thioether in two ways (*15*). In either case the presence of additional hydrocarbons (Fig. 1A) of molecular weight 276 (peak 3, mass spectrum shown in Fig. 1C; peak 4, two species whose spectra are not shown; that of the major species is essentially identical to that of peak 3) would be consistent with partial hydrogenation by Raney nickel of the two $C_{20}H_{34}$ compounds, respectively.

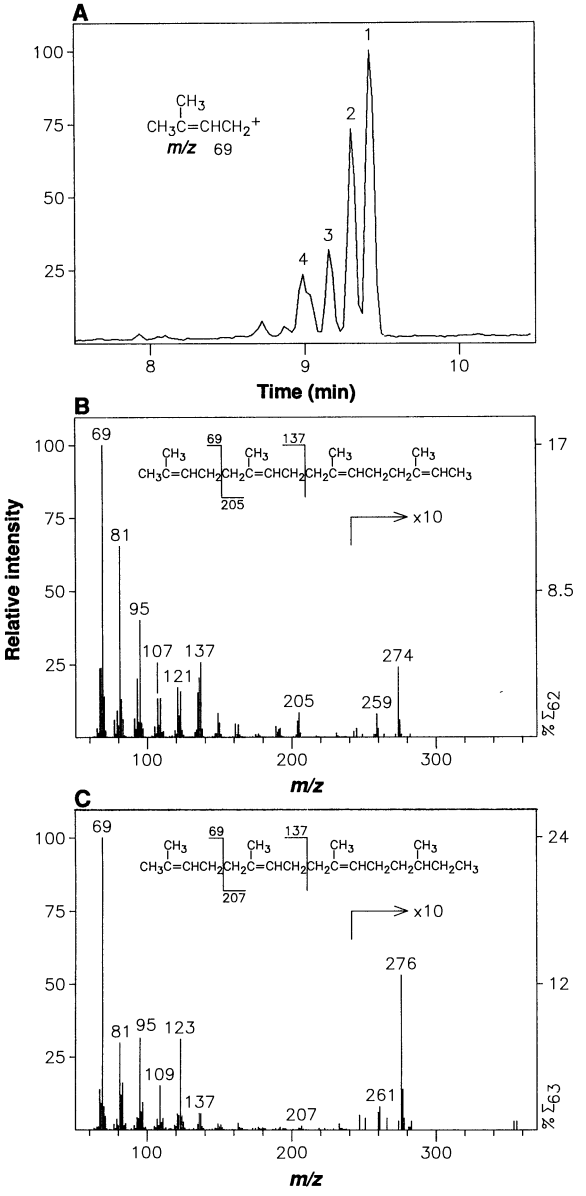

Fig. 1. Gas chromatography–mass spectrometry (*16*) of products of Raney nickel desulfurization of prenylated cysteines released by hydrazinolysis of total proteins from CHO-K1 cells. (**A**) Reconstructed ion chromatogram for the characteristic isoprene ion, *m/z* 69; no other significant isoprenoid products were observed. (**B**) Mass spectrum of peak 1. (**C**) Mass spectrum of peak 3. Structures accompanying the mass spectra are arbitrarily drawn to simplify presentation, and are not meant to imply that double-bond geometry is known.

Although our evidence does not permit the assignment of double-bond geometry, it is most reasonable to assume that three double bonds are E, which is consistent with most known terpenes from higher animals. A definitive assignment of structure must await further studies. Nonetheless, this prenyl group has been isolated from higher animal tissues as a product of de novo synthesis and has been established as being covalently attached to protein, as opposed to the case for fungal prenylated peptides. Hancock *et al.* (*2*) reported that protein from COS cells was covalently modified by farnesol- and geraniol-like compounds. However, it is doubtful that the treatment they used, digestion with pronase, cleaved the thioether bond of the prenyl cysteine. The relatively nonpolar material that they found on reversed-phase thin-layer chromatography was likely a prenyl cysteine.

The carboxyl-terminal cysteine of prenylated Ras protein and yeast mating factor occurs as the methyl ester. The prenylated proteins of CHO cells are not so methylated. Hydrazinolysis of esters of amino acids would yield hydrazides, which would be retained on ion-exchange chromatography during isolation of the hydrolysis products. Since we recovered nearly all of the radioactive prenylated material in the neutral amino acid fraction, most, if not all, of the proteins have a free terminal carboxyl group. Thus CHO cells either do not require esterification capability or we have isolated incompletely processed prenylated proteins.

Perhaps the function of the prenyl group of these cytosolically synthesized proteins is to anchor them in membranes; Hancock *et al.* (*2*) have shown that palmitoylation, although not essential for activity, does enhance the binding. Furthermore, the C_{20} diterpene can be looked upon as a C_{16} straight chain with four side chain methyl groups and thus is similar to the 16 carbon atoms of the palmitoyl group.

REFERENCES AND NOTES

1. W. R. Schafer *et al.*, *Science* **245**, 379 (1989).
2. J. F. Hancock, A. I. Magee, J. E. Childs, C. J. Marshall, *Cell* **57**, 1167 (1989).
3. E. Santos and A. R. Nebreda, *FASEB J.* **3**, 2151 (1989).
4. R. A. Schmidt, C. J. Schneider, J. A. Glomset, *J. Biol. Chem.* **259**, 10175 (1984).
5. E. Bruenger and H. C. Rilling, *Biochem. Biophys. Res. Commun.* **139**, 209 (1986).
6. W. A. Maltese and K. M. Sheridan, *J. Biol. Chem.* **263**, 10104 (1988).

7. J. A. Beck, T. J. Hosick, M. Sinensky, *J. Cell Biol.* **107**, 1307 (1988).
8. R. J. Anderegg, R. Betz, S. A. Carr, J. W. Crabb, W. Duntze, *J. Biol. Chem.* **263**, 18236 (1988).
9. A. Kamiya *et al.*, *Agric. Biol. Chem.* **43**, 363 (1979).
10. H. C. Rilling, E. Bruenger, W. W. Epstein, A. A. Kandutsch, *Biochem. Biophys. Res. Commun.* **163**, 143 (1989).
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.
12. H. Frankel-Conrat and C. M. Tsung, *Methods Enzymol.* **11**, 151 (1967).
13. B. R. Pettit and E. E. Van Tamelen, *Org. Reactions* **12**, 356 (1962).
14. C. R. Enzell, R. A. Appleton, I. N. Wahlberg, in *Biochemical Applications of Mass Spectrometry*, G. R. Waller, Ed. (Wiley-Interscience, New York, 1972), pp. 351–385.
15. P. N. Rylander, *Catalytic Hydrogenation in Organic Synthesis* (Academic Press, New York, 1979), pp. 289–290.
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.
17. Supported by the University of Utah Research Committee, NIH grant GM 29812 (P.F.C.), and in part by NSF grant DCB 88-03825 (H.C.R.).

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Identification of Geranylgeranyl-Modified Proteins in HeLa Cells

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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-

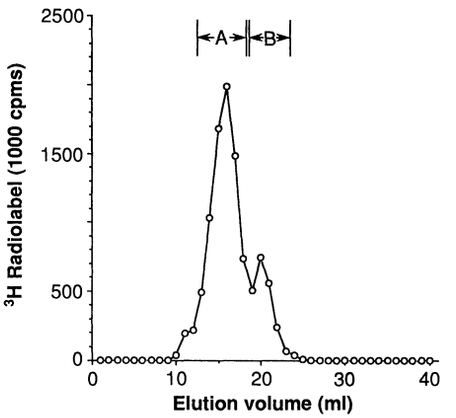

Fig. 1. Size-exclusion chromatography of proteolytic hydrolysates of HeLa cell total proteins on Sephadex LH-20. Cells were labeled for 36 hours with [5-^{3}H]mevalonic acid in the presence of 30 μ*M* mevinolin, harvested, washed with phosphate-buffered saline, and extracted with lipid solvents. Cell 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.

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