(pore sizes of 0.2, 1, 3, 12, 20, 41, 200, and 400 μm) were plotted against the logarithm of filter size. The slope of this linear relation was used as the index of plankton size distribution [see (*14*) for details].
18. Heat content of lakes was calculated for each date from volume-weighted mean temperature for each meter depth stratum [G. A. Cole, *Textbook of Limnology* (Mosby, London, 1979)]. Temperature measured at the center of the enclosures was used for the calculation.
19. Fetch is the distance in a water body over which wind can blow uninterrupted by land (*3*). It is equal to the square root of surface area.
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Phenotypic Switching in Mycoplasmas: Phase Variation of Diverse Surface Lipoproteins

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The ability of some microorganisms to rapidly alter the expression and structure of surface components reflects an important strategy for adaptation to changing environments, including those encountered by infectious agents within respective host organisms. *Mycoplasma hyorhinis*, a wall-less prokaryotic pathogen of the class Mollicutes, is shown to undergo high-frequency phase transitions in colony morphology and opacity and in the expression of diverse lipid-modified, cell-surface protein antigens. These proteins spontaneously vary in size, contain highly repetitive structures, and are oriented with their carboxyl-terminal region external to the membrane. Thus, mycoplasma membrane lipoproteins generate microbial surface diversity and may be part of a complex system that controls interactions of these organisms with their hosts.

SEVERAL MICROBIAL SPECIES SHOW reversible, high-frequency changes in phenotype, including oscillating expression (phase variation) or antigenic diversification of surface macromolecules (*1, 2*). In many instances, components associated with phase variation in bacteria are controlled by complex regulatory systems that affect both physiologic processes and virulence of the organisms (*1*).

We have investigated the presence of antigenic and phase variation in *Mycoplasma hyorhinis*, one of over 70 species in the genus *Mycoplasma*. Like several members of this group, *M. hyorhinis* is an animal pathogen. It causes rhinitis, polyserositis, and chronic experimental arthritis in its natural swine host (*3, 4*). This agent is also a common contaminant of tissue cell cultures where it propagates as a cell-surface parasite (*5*). Isolates of *M. hyorhinis* differ with respect to pathogenic potential (*4*), the ability to adsorb to cells in vitro (*6*), requirements for independent growth in agar medium (*7*), and antigenic characteristics (*8*), including marked differences in the expression and size of lipid-modified, integral membrane protein antigens at the cell surface that are recognized by monoclonal antibodies (*9*). It is important to determine whether phenotypic differences within this species arise from accumulated mutational events specifying particular characteristics of an isolate or, rather, reflect intrinsic, heritable mechanisms for generating diversity within populations in order to understand the interactions of this species, and perhaps several other pathogenic mycoplasmas, with their respective hosts. For this reason, we analyzed *M. hyorhinis* strain SK76, an arthritogenic swine pathogen (*10*) obtained as a multiply filter-cloned isolate, for its ability to diversify phenotypically during independent, cell-free growth in vitro.

Early and late (over 100) passage broth cultures of *M. hyorhinis* showed consistent heterogeneity in colony morphology when plated on standard agar medium (Fig. 1, A to D). A stable proportion of three "morphotypes" (indicated as a percentage of the colonies plated from filtered broth culture) was independent of the number of passages: a small-diameter S morphotype with a prominent center (98.6%), an intermediate-size M morphotype with a flat appearance and rough surface (1.3%), and a large-diameter L morphotype with a hemispherical rounded shape and smooth surface (0.1%). Several rounds of single colony isolation and filter cloning yielded purified cultures of each morphotype (Fig. 1, A to C), which were easily distinguished when plated as a mixture (Fig. 1D). However, close examination of subcloned populations ("lineages"), derived from serial rounds of single colony isolations, showed switching of each morphotype to both of the other morphotypes. Further analysis of lineages derived from "switched" progeny showed additional switching, either reverting to the original or again generating alternate phenotypes. Oscillating morphotypic switches of L, S, and M morphotypes were quantitatively monitored in several lineages (n) with the following ranges of switch frequencies (fraction of switched phenotype per cell per generation) (*11*): S → L [(0.1 to 43) × 10^{-4}, $n = 6$]; L → S [(0.7 to 2.9) × 10^{-4}, $n = 5$]; S → M [(0.7 to 64) × 10^{-4}, $n = 10$]; M → S [(1.0 to 6.2) × 10^{-4}, $n = 8$]; L → M (0.2 × 10^{-4}, $n = 1$); and M → L [(1.3 to 4.8) × 10^{-4}, $n = 3$]. Mean frequencies for these transitions ranged from 0.2×10^{-4} to 14.4×10^{-4}. Extremely high switch frequencies (~3×10^{-2}, the maximum measurable) were apparent in some lineages, where virtually every colony plated showed one morphotype spawning "sectors," subsequently shown to represent a switch to a different morphotype (Fig. 1, inset).

A second phenotype showing diversity in *M. hyorhinis* populations was colony opacity. Subcloned lineages of each morphotype showed heterogeneity in this feature, which was therefore not linked to colony morphology. Lineages of L variants (the most easily observed morphotype) generated oscillating transparent (tr) or opaque (op) populations (Fig. 1, E to G) with a high frequency of switching with respect to this characteristic: tr → op [(0.3 to 74) × 10^{-4}, $n = 8$] and op → tr [(3.2 to 93) × 10^{-4}, $n = 17$]. In several additional lineages, sectoring was observed in all colonies plated. The ability to undergo reversible high frequency, independent switching of colony morphotype, and opacity was a heritable feature of the organism.

An additional phenotypic instability was identified in subcloned *M. hyorhinis* populations by immunostaining colonies with the monoclonal antibody (MAb) F192C17a to assess expression of corresponding epitope-bearing lipoproteins (*9*). Unlike the uniform immunostaining of colonies obtained with polyclonal antibodies to *M. hyorhinis* or the MAb AB3C to p70 (*12*), a strain-invariant surface protein of this species (*9*), the staining obtained with MAb F192C17a was

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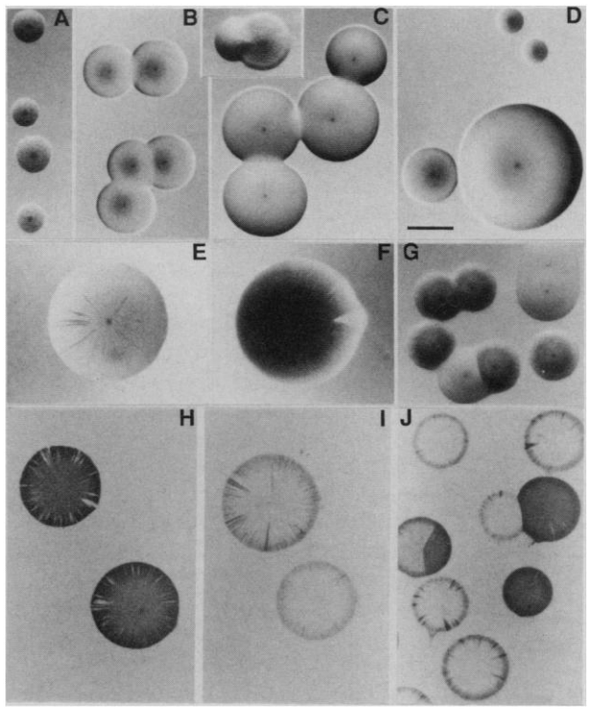

Fig. 1. Variation of colony morphology, opacity, and surface antigen expression in subcloned lineages of *M. hyorhinis* strain SK76. Lineages were derived by serial rounds of filter-cloning (*19*). The colonies were derived from filtered populations that were plated directly after expansion in broth culture from single-colony parents (*20*). (**A** to **D**) Colonies from multiply subcloned lineages purified by selection for the S (A), M (B), or L (C) morphotypes. (D) A mixture of these three purified populations shown on a single plate; the bar indicates 0.5 mm. (**Inset**) A sector emerging from an M-type colony, subsequently shown to be an L-type variant. (**E** to **G**) Colonies from multiply subcloned lineages of the L morphotype showing dark opaque (op) sectors in a light, transparent (tr) colony (E), tr sectors in an op colony (F), and a population derived from a sectored colony showing both op and tr progeny (G). (**H** to **J**) Colonies transferred to nitrocellulose filters and immunostained with MAb F192C17a, which recognizes a set of size-variant surface lipoprotein antigens of *M. hyorhinis* (*9*). Representative immunostained colonies from multiply subcloned lineages exhibiting negative (H) or positive (I) sectors within colonies of predominantly the opposite antigenic phenotype, and variation in colony staining within a population derived directly from a single-colony parent with sectored immunostaining (J) are shown.

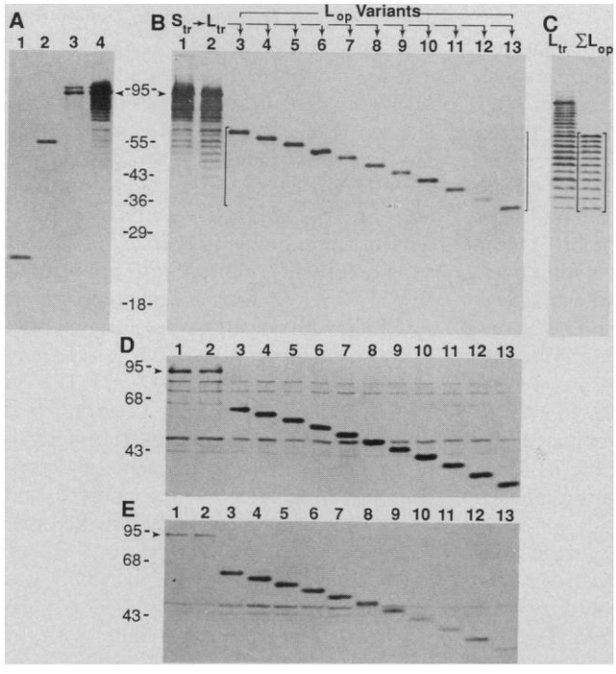

Fig. 2. Generation of size-variant membrane lipoprotein antigens during phenotypic phase transitions in subcloned lineages of *M. hyorhinis* strain SK76 (*21*). (**A** to **C**) Immunoblots with MAb F192C17a of mycoplasma proteins separated by SDS-PAGE. (A) Equal quantities of mycoplasmas (~2×10^7 CFU) from continuously passaged logarithmic-phase broth cultures of strains GDLI (lane 1), GDLII (lane 2), or SK76 (lane 3); or a tenfold greater load of SK76 (lane 4). (B) Populations in a direct lineage selected by serial subcloning of single antigen-positive colonies representing sequential transitions (indicated by arrows) from a multiply subcloned transparent S variant (S_{tr}, lane 1); to a transparent L variant (L_{tr}, lane 2); to several opaque L variants (L_{op}, lanes 3 to 13), indicated by brackets. Samples contained approximately 2×10^8 (lanes 1 and 2) or 2×10^7 (lanes 3 to 13) CFU. (**C**) Comparison of the immediate progenitor L_{tr} population (~2×10^8 CFU) to a mixture of individual progeny (ΣL_{op}) representing equal proportions (~6×10^6 CFU each) of the 11 variants (brackets) shown in (B), lanes 3 to 13. (**D** and **E**) Fluorographs of Triton X-114 phase-separated proteins from populations corresponding exactly to those in (B), and labeled with [^{35}S]cysteine (D) or [^{3}H]palmitate (E). Molecular mass markers are shown in kilodaltons. Arrows indicate the position of the predominant 90-kD lipoprotein antigen recognized by MAb F192C17a in SK76 populations displaying heterogeneous size-variant antigens.

heterogeneous both in populations derived from single colony isolates and within individual colonies, most of the latter showing clear sectoring by this method (Fig. 1, H to J). Subcloned populations oscillating between expression (+) or absence (−) of this epitope showed high switch frequencies (+ → − or − → +) of (2.1 to 47) × 10^{-4} (n = 7), these values again representing minima because some sectoring was often seen even in the most uniformly immunostained (or unstained) colonies. Sectored immunostaining was observed in all morphotypes and occurred both in op and tr colonies. Although this trait could therefore vary independently, concomitant switches of two phenotypes (for example, $op^+ \rightarrow tr^-$) were often observed. High-frequency phenotypic switching of colony morphology, opacity, and F192C17a epitope expression occurred throughout this species. Marked heterogeneity of these features was observed in previously described clonally purified strains (*9*) and in primary passages from filter-cloned American Type Culture Collection (Rockville, Maryland) stocks of *M. hyorhinis*: ATCC 23839 (GDL); 25021, 25026, and 25077 (PG29); 17981 (BTS-7); and 27717 (a subcloned BTS-7 stock derived from 17981) (*12*).

The isolation of subcloned populations showing phase variation in surface epitope expression allowed SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the corresponding lipoprotein expression and size variation originally described in several strains of *M. hyorhinis* (*9*). Strain SK76 expressed a heterogeneous "ladder" of amphiphilic, size-variant surface lipoproteins bearing the epitope, which differed from the more restricted, single size variants expressed by other strains (*9*) (Fig. 2A). This heterogeneous pattern was maintained in several subcloned lineages of the SK76 strain, even during transitions between morphotypes (Fig. 2B, lanes 1 and 2). In contrast, analysis of some lineages revealed marked variation in the size of lipoprotein antigens that was manifest as several discrete forms, each expressed separately on individual clones undergoing tr → op phase transitions. Such a transition was observed in a population derived from a single L_{tr}^+ colony that was shown to express the complete set of size-variant antigens (Fig. 2B, lane 2). L_{op}^+ variants were isolated from this same population (at a frequency of 58 × 10^{-4}) that expressed distinct size variants of the antigen (Fig. 2B, lanes 3 to 13). From 60 of these L_{op}^+ variants analyzed by SDS-PAGE, 11 different size-variant antigens were detected, which collectively accounted for a large portion of the heterogeneous "ladder" in the progenitor L_{tr}^+ population (Fig. 2C).

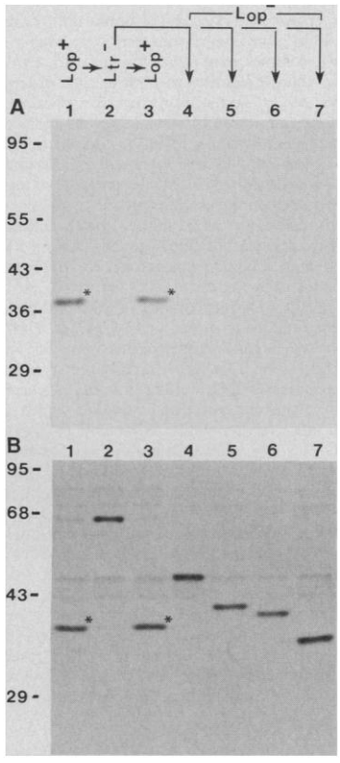

Fig. 3. Phase transitions involving expression and structural variation of *M. hyorhinis* membrane lipoproteins. Subcloned populations of strain SK76 were either subjected to SDS-PAGE and immunoblotted with MAb F192C17a (**A**) or were labeled with [^{35}S]cysteine and the Triton X-114–phase proteins analyzed by fluorography (**B**), as described (Fig. 2). Populations from a direct lineage of L morphotype were selected for subcloning by colony immunoblotting with MAb F192C17a and represent sequential transitions (indicated by arrows) from an epitope-positive opaque variant [identical to the variant in Fig. 2B (lane 12)] (L^{+}_{op}, lane 1), to an epitope-negative transparent variant (Ltr^{-}, lane 2), and from this variant to a subsequent revertant L^{+}_{op} variant (lane 3) or to additional epitope-negative opaque (L^{-}_{op}) variants (four of which are represented in lanes 4 to 7). Asterisks indicate proteins recognized by MAb F192C17a. Molecular mass markers are shown in kilodaltons. Identical populations are represented in the corresponding lanes in (A) and (B).

Analysis by SDS-PAGE of large amounts of these discrete size variants showed only minor descending "ladders," indicating relative stability in the size within each variant line (*12*). These spontaneously occurring size variants showed uniform spacing on SDS-polyacrylamide gels (under reducing conditions) corresponding to a difference in apparent molecular mass of ~2.8 kD (Fig. 2, B and C). In addition, the variants could be labeled metabolically with [^{35}S]cysteine (Fig. 2D) or [^{3}H]palmitate (Fig. 2E), but not with [^{35}S]methionine (*12*), and they all partitioned as amphiphilic integral membrane proteins during Triton X-114 phase-fractionation (*9*) (Fig. 2, D and E). Analysis of clonal lineages therefore established that mechanisms also operate in these organisms to generate antigen size variation at high frequency in isogenic populations.

We analyzed phase variation in expression of lipoprotein surface antigens in subcloned lineages selected for oscillating expression of antigen by colony immunostaining. We observed a transition in one lineage, representing the reversible switch: $L^{+}_{op} \rightarrow L^{-}_{tr} \rightarrow L^{+}_{op}$ (Fig. 3, lanes 1 to 3). Expression of 37-kD epitope-bearing lipoprotein corresponded precisely to the expression pattern in colony immunostaining. However, the epitope-negative intermediate (L^{-}_{tr}) expressed an alternative amphiphilic protein that could be labeled with cysteine (Fig. 3B, lane 2) and palmitate (*12*), but not with methionine (*12*), and yet lacked the epitope recognized by the MAb (Fig. 3A, lane 2). This intermediate gave additional progeny expressing a series of discrete, size-variant amphiphilic lipoproteins, all of which lacked this epitope (Fig. 3A, lanes 4 to 7) and had similar labeling properties (Fig. 3B, lanes 4 to 7). The presence of a structurally analogous, alternative set of size-variant lipoproteins, antigenically distinct from those recognized by MAb F192C17a in the immediate progenitor population, established that high-frequency phase variation of specific lipoprotein antigens, as well as antigenic variation within a group of biochemically similar lipoproteins, occurred in clonally derived populations of this species. Alternative, secreted forms of epitope-bearing proteins were not detected during phase transitions (*12*). Several lineages examined [including series generated from all 11 single size variants in Fig. 2B (lanes 3 to 13)] showed both antigenic and phase variations in these lipoproteins. The ability to oscillate between expression of any set of size-variant lipoprotein antigens recognized by this MAb was further established by analysis of a lineage originating from an L^{+}_{op} variant with a single antigenic size form. This variant gave rise, through an epitope-negative intermediate, to an L^{+}_{tr} variant population that again expressed the entire repertoire of size variants (*12*).

The distinctive structural features and orientation of variant surface lipoproteins were initially characterized by partial proteolytic digestion of intact organisms representing L^{+}_{op} variants [from the series shown in Fig. 2B (lanes 3 to 13)] that predominantly expressed individual epitope-bearing lipoprotein size variants. Partial digestion of these clones with trypsin (which cleaves predominantly at Lys and Arg residues) generated a ladder of epitope-bearing proteins from all 11 size variants, two of which are shown in Fig. 4A. Enzymatically generated fragments showed size differences identical to those observed in the naturally occurring ladder in this lineage (Fig. 2C), with a minimum size of about 23 kD. Complete digestion yielded no resolvable products that bound the MAb F192C17a. Digestion of these variant clones with carboxypeptidase Y (Fig. 4B) [which successively cleaves COOH-terminal amino acid residues and hydrolyses Lys, Arg, and His residues at greatly reduced relative rates (*13*)] yielded a ladder of epitope-bearing products with the same spacing, and a minimum size of ~26 kD. This result suggested that most of the COOH-terminal portion of the lipoproteins is accessible at the external surface of the organism and that it contains a periodic

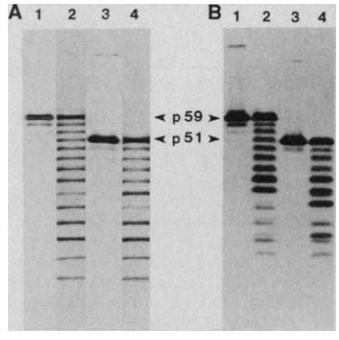

Fig. 4. Proteolytic analysis of lipoprotein antigen surface-orientation and periodic structure. Immunoblots with MAb F192C17a represent two subcloned L^{+}_{op} variants derived from the same parent colony, expressing a 59-kD antigen (p59, lanes 1 and 2) or a 51-kD antigen (p51, lanes 3 and 4), which correspond to those in Fig. 2B (lanes 4 and 7, respectively). Organisms were incubated without enzyme (lanes 1 and 3) or with varying amounts of trypsin (**A**) or carboxypeptidase Y (**B**) (lanes 2 and 4). Arrows show position of the 59- and 51-kD antigens in the two variants. (A) and (B) represent blots from separate SDS-polyacrylamide gels. L^{+}_{op} variants expressing single, predominant sizes of lipoprotein antigen [as in Fig. 2B (lanes 3 to 13)] were grown in broth culture, harvested, and incubated in phosphate-buffered saline containing either no enzyme or fivefold increasing concentrations of TPCK (*L*-1-tosylamide-2-phenyl-ethylchloromethyl ketone)–trypsin (Sigma; 0.08 to 2 μg/ml, *p*H 8.0) or carboxypeptidase Y (Boehringer Mannheim; 0.08 to 2 mg/ml, *p*H 6.0) at 37°C for 1 hour. Entire digestion mixtures were analyzed immediately by SDS-PAGE, followed by immunoblotting with MAb F192C17a as described (Fig. 2). After photography, blots were restained with MAbs F24C4Fg and F81C39R (*9, 15*), which recognize, respectively, p38 and p51 internal proteins as a control to ensure that cells remained impermeable to proteolytic enzymes during digestions (*12*). Digestion mixtures obtained with varying concentrations of each enzyme were pooled to fully represent all of the size-variant forms of the epitope-bearing digestion products shown in lanes 2 and 4.

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*).

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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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