Characterization of Isoprenoid Involved in the Post-translational Modification of Mammalian Cell Proteins*

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Mammalian cell proteins, modified post-translationally by derivatives of [3H]mevalonic acid, were subjected to methylation and sulfonium salt cleavage reactions previously used to release isoprenoids from cysteine residues in yeast peptides. The labeled isoprenoid extracted into chloroform comigrated with farnesol through a series of chromatography steps including Sep-Pak C-18 fractionation, size exclusion on Bio-Beads, and reverse-phase chromatography. Further resolution of the material by normal phase liquid chromatography and thin layer chromatography demonstrated the presence of farnesol, norvaliol, and other unidentified hydrophobic derivatives. Similar products were generated when S-farnesyl cysteine was subjected to the methylation and cleavage procedures. These preliminary findings suggest that farnesylation of cysteine residues accounts for the well documented incorporation of mevalonic acid into mammalian cell proteins.

Several recent reports have described a novel type of post-translational modification which involves the incorporation of isoprenoid derivatives of mevalonic acid (MVA) into several mammalian cell proteins (1–11), including the nuclear lamins (5, 6) and α2M (11). This modification has been referred to by the general term “isoprenylation” because of the lack of structural information about the isoprenoid groups involved. Post-translational modification of proteins with isoprenoid groups is not unique to mammalian systems. In fact, the modification of fungal mating peptides with isoprenoids has been studied in considerable detail. It is now known that these peptides are modified by farnesol, linked to cysteine via a thioether bond (12–16). Hypothesizing that the isoprenoid modification of mammalian cell proteins might be analogous to that found in peptides derived from lower eukaryotes, we used procedures similar to those used in the studies of mating peptides to remove radiolabeled isoprenoids from proteins in cultured murine erythroblastic leukemia cells. Herein we report a preliminary chromatographic characterization of this material which suggests that proteins in cultured mammalian cells are modified by farnesy1 residues.

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2 The abbreviations used are: MVA, mevalonic acid; MEL, murine erythroblastic leukemia; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Material—R,S-[3H]Mevalonolactone (3.5 Ci/mmol) was prepared by reduction of mevalonic acid precursor (Sigma) with sodium borohydride (5 Ci/mmol, Amersham Corp.) as described by Keller (17). Purity of the tritium-labeled product was checked by TLC as described previously (6). Lovastatin (mevinolin), a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, was a gift from Alfred W. Alberts (Merck, Sharp and Dohme Research Laboratories), tissue culture medium and serum (GIBCO/Bethesda Research Laboratories), tissue culture flasks (Corning), and HPLC grade organic reagents (J. T. Baker Chemical Co.) were obtained from the designated sources. Cholesterol, dolichol, ubiquinone-10, and squalene standards for chromatography were obtained from Sigma. Geraniol, norvaliol, and farnesol were purchased from Aldrich.

Cell Culture—Murine erythroblastic leukemia cells (line 745) were a gift from Dr. Charlotte Friend (Mt. Sinai School of Medicine, New York). Cells were maintained in suspension culture as described previously (7). For all of the studies described in this report we used a lovastatin-resistant MEL cell line (LR-MEL) that was adapted to grow continuously in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum and 25 μM lovastatin.

Removal of Isoprenoids from Proteins Labeled with [3H]MVA—Cultures of LR-MEL cells were incubated with radiolabeled MVA under various conditions, as described in the figure legends. At the end of the incubation period, cells were pelleted by centrifugation, washed three times with ice-cold phosphate-buffered saline, and homogenized in 5 volumes of buffer containing 25 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 0.1 μM pepstatin. The soluble protein was obtained by centrifuging the homogenate at 100,000 x g for 90 min at 4 °C. Protein was precipitated by the addition of 10% (v/v) acetone and collected by centrifugation. The precipitate was extracted successively with petroleum ether (twice), ethyl acetate, and acetone to remove noncovalently bound lipids.

Removal of [3H]MVA-labeled isoprenoid from protein was accomplished by forming the S-methylsulfonium derivative with CH3I and subsequently cleaving the sulfonium salt to release the free isoprenoid. The procedures were patterned after those used to extract farnesyl groups from cysteine residues of fungal mating factors (12, 14, 16). For studies aimed at determining whether the protein-isoprenoid bond was labile to CH3I (Fig. 1), the delipidated protein precipitate (0.4–0.5 mg of protein) was dissolved in 1.4 ml of solution containing 8 M urea, 2% (w/v) SDS, 10% glycerol, and 3% formic acid. After the addition of 0.55 ml of CH3I (Aldrich), the mixture was shaken at 37 °C in a covered reciprocating water bath for 24 h. The residual CH3I was removed under a vacuum, and the pH of the solution was adjusted to 10.3 by the addition of approximately 140 μl of 10 N NaOH. The incubation was continued for an additional 20 h, and the protein was finally precipitated with 10% trichloroacetic acid. SDS-PAGE and fluorography of the proteins were carried out as described previously (7, 8). Controls consisted of protein samples which were treated identically, except that CH3I was omitted during the first incubation. For all other studies in which the aim was to characterize the isoprenoid released from the protein, the delipidated protein precipitate (0.5–2.3 mg) was suspended in 1.4 ml of 3% formic acid, 0.55 ml of CH3I was added, and the same procedure was followed. However, at the end of the second incubation, instead of precipitating the protein with trichloroacetic acid, 2 ml of methanol was added to the reaction mixture followed by 4 ml of chloroform. After brief centrifugation to facilitate phase separation, the chloroform phase was collected and the solvent was evaporated under a stream of N2.
The residue was dissolved in 100–200 μl of methylene chloride and analyzed as described below.

Reverse-phase Chromatography—Crude fractionation of the labeled isoprenoid material extracted from the proteins was accomplished by loading it on a Sep-Pak C-18 cartridge (Waters Associates, Inc.) which was eluted sequentially with 5 ml of methanol, 5 ml of acetone, and 5 ml of methylene chloride as described by Wong and Lennarz (18). The methanol fraction, which contained most of the radioactivity, was concentrated by evaporation under N₂. Reverse-phase HPLC was carried out on a Beckman System Gold chromatograph equipped with a Beckman C-18 Ultrasphere column (5 μm, 4.6 × 150 mm). Isoprenoids were eluted at 1 ml/min with a linear gradient of 20–80% (v/v) isopropyl alcohol in methanol over 20 min, followed by isocratic elution with 80% isopropyl alcohol in methanol for 10 min. Fractions were monitored by their absorbance at 210 nm and by counting aliquots of the eluate in a liquid scintillation spectrometer.

Normal Phase Chromatography—Radioactive fractions from the reverse-phase HPLC column were pooled and concentrated by evaporation under N₂. Normal phase HPLC was then carried out using a Bakerbond silica gel column (5 μm, 4.6 × 250 mm) eluted isocratically at 1 ml/min with hexane/isopropyl alcohol (97:3, v/v). Thin layer chromatography was performed on Brinkmann plastic-backed Silica Gel G plates (0.25-mm thickness) using a solvent system of hexane/diethyl ether/glacial acetic acid (70:30:1.5, v/v/v). Isoprenoid standards were visualized by spraying the thin layer plates with anisaldehyde reagent (19).

Gel Exclusion Chromatography—An aliquot of the radioactive material eluted from the Sep-Pak column with methanol was mixed with isoprenoid standards, dried under N₂, dissolved in methylene chloride, and applied to a 0.5 × 30.0-cm column of Bio-Beads S-X3 (200–400 mesh). The column was eluted with methylene chloride at a flow rate of 0.4 ml/min. The elution position of the standards was determined by spotting samples of the eluate onto thin layer plates and spraying with anisaldehyde. Radioactivity was monitored by liquid scintillation counting.

Synthesis of S-Farnesyl Cysteine—Farnesyl bromide was prepared from trans-trans farnesol and N-bromosuccinimide as described by Corey et al. (20). Synthesis of S-farnesyl cysteine was then carried out as described by Kamiya et al. (21) with the addition of 4 × 10⁶ dpm of [³⁵S]cysteine (Amersham Corp., 1007 Ci/mmole) as a tracer. The product was purified by thin layer chromatography on Silica Gel G plates developed with the upper phase of a mixture of 1-butanol/ethylene hydrolysis. A strip of the plate was stained with anisaldehyde, then cut into segments and counted for radioactivity. Farnesyl cysteine appeared as an anisaldehyde-positive, [³⁵S]cysteine-labeled zone, with an Rf of 0.68, which was well resolved from the unreacted farnesol migrating at the solvent front. Other free isoprenoids (e.g., nerolidol, geraniol) also move with the solvent front in this system.

Farnesyl cysteine was eluted from the silica gel with 1-butanol and stored under N₂, in the dark at -20 °C. Chromatography of an aliquot of the material using a second solvent system (1-butanol/ethanol/pyridine/water, 15:3:10:12) yielded a single anisaldehyde-positive cysteine-labeled zone with an Rf of 0.85. The Rf values of the synthetic material in both of the TLC systems (0.68 and 0.85) were the same as those previously reported for farnesyl cysteine in the same chromatography systems (21).

RESULTS

The SDS-PAGE fluorographic profile of the Triton X-100-soluble proteins from [³⁵H]MVA-labeled LR-MEL cells was similar to that previously seen in homogenates from a variety of cultured cell lines (1–4, 7, 8) insofar as most of the radioactivity was incorporated into a cluster of proteins between 21 and 28 kDa. When the delipidated protein was treated with CH₃J prior to electrophoresis, most of the [³⁵H]MVA-derived radioactivity associated with the proteins was lost (Fig. 1B). Incubation of the proteins under identical conditions without CH₃J had no substantial effect on the pattern of labeled proteins (Fig. 1A). The profiles of stained protein bands in both gels were similar (Fig. 1, C and D), suggesting that the absence of discrete bands of radioactivity in the CH₃J-treated sample was due to release of isoprenoid groups from the polypeptides rather than general protein hydrolysis or denaturation.

**TABLE I**

<table>
<thead>
<tr>
<th>Elution fraction</th>
<th>Disintegrations/min radioactivity</th>
<th>Total radioactivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>450,000</td>
<td>89</td>
</tr>
<tr>
<td>Acetone</td>
<td>30,000</td>
<td>6</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>15,000</td>
<td>3</td>
</tr>
<tr>
<td>Sep-Pak residual</td>
<td>11,500</td>
<td>2</td>
</tr>
</tbody>
</table>

In a separate study aimed at identifying the isoprenoid groups released by CH₃J treatment, the protein was extracted with chloroform/methanol (2:1) after methylation and sulfonium salt cleavage. Under these conditions, 86% of the [³⁵H] MVA-derived radioactivity was extracted into the chloroform phase whereas only 13% of the radioactivity was extracted into chloroform when CH₃J was omitted from the reaction mixture. The first step in our characterization of the chloroform-extractable material released from the protein involved a simple fractionation scheme using a Sep-Pak C-18 column. As shown in Table 1, 86% of the radioactivity recovered from the CH₃J-treated protein was eluted from the column with methanol. When isoprenoid standards were subjected to this procedure, the short and medium chain polyprenols (geraniol, farnesol) were eluted with methanol, whereas the more hydrophobic long chain polyprenols (dolichol, ubiquinone-10) were eluted later with the acetone wash. To establish the relative size of the labeled isoprenoid, an aliquot of the methanol eluate from the Sep-Pak column was subjected to gel exclusion chromatography on Bio-Beads (Fig. 2A). The peak of radioactivity comigrated with the farnesol standard and was well resolved from the shorter chain (geraniol) and the longer chain (dolichol) standards. A more detailed analysis of the chromatographic behavior of the radioactive material eluted from the Sep-Pak column was carried out by reverse-phase HPLC. As shown in Fig. 2B, most of the radioactive material derived from the modified proteins was eluted near the position of the farnesol standard (peaks 1 and 2).
Isoprenoid Modification of Proteins

Previous studies of yeast and fungal mating factors have revealed that their modifying farnesyl groups undergo a number of complex rearrangements during the methylation and cleavage reactions used to release the isoprenoid from cysteine residues (12, 14, 16). The products known to be generated in addition to farnesol include nerolidol and cyclic tertiary alcohols such as \( \alpha \)-bisabolol. Since these products are not readily resolved by reverse-phase chromatography, we subjected each of the radioactive peaks from the C-18 HPLC column (Fig. 2B) to further analysis by normal phase chromatography. Fig. 2C shows that when the material in the major radioactive peak (peak 1) from the C-18 column was subjected to HPLC on a silica column, two distinct isoprenoid species were separated, one comigrating with farnesol and the other comigrating with nerolidol. Further confirmation of the identities of these two compounds was obtained by chromatographing an aliquot of the sample on silica gel thin layer plates with a different solvent system (Fig. 3). The trailing

peaks from the reverse-phase HPLC column (Fig. 2B, peaks 2, 3 and 4) were also subjected to TLC. In contrast to peak 1, which yielded farnesol and nerolidol, each of the trailing peaks gave rise to a single major zone of radioactivity near the solvent front of the TLC plates (Fig. 3). Although the squalene standard also moved to this position on the TLC plates, the radioactive material clearly was not squalene, based on the results of HPLC (see Fig. 2B). Thus, the material in peaks 2, 3 and 4 may correspond to hydrophobic cyclic rearrangement products derived from farnesol (e.g. \( \alpha \)-bisabolol). To test this possibility, methylation and cleavage of \( S \)-farnesyl cysteine were carried out and the chloroform-extractable products were
chromatographed along with a sample of radioactive isoprenoid material derived from cell protein. As shown in Fig. 4, only a relatively small part of the anisaldehyde-stained material released from farnesyl cysteine (lane B) migrated as authentic farnesol (lane A). Most of the material migrated as a broad zone near the solvent front and as a double spot in the region of the nerolidol standard. It is noteworthy that farnesol did not give rise to multiple products when the free isoprenoid was subjected to incubation with CH$_3$I (not shown). Thus, the generation of rearrangement products appears to occur specifically in conjunction with the cleavage of the farnesyl cysteine thioether. When the TLC plate was sectioned and counted, the three major zones of radioactivity derived from the $[^3]$H]MVA-labeled protein coincided with the three major anisaldehyde-stained products generated from farnesyl cysteine (Fig. 4). Of the total radioactivity overlapping the major anisaldehyde-stained zones, 23% comigrated with farnesol, 38.7% with nerolidol, and 38.4% with the unidentified material near the solvent front. Therefore, the distribution of radioactivity in the products released from the proteins resembled that of the stained products released from farnesyl cysteine insofar as farnesol was the least abundant component of the mixture. The non-farnesol radioactivity derived from the proteins was about equally distributed between nerolidol and the material at the solvent front, whereas the latter isoprenoid appeared to predominate in the farnesyl cysteine extract. This discrepancy could be due to differences in the rearrangements that occur when farnesol is cleaved from a protein rather than from free farnesyl cysteine, or it might reflect differences in the staining properties of the isoprenoid products (e.g. nerolidol versus the unidentified material) with anisaldehyde.

To verify that the $[^3]$H]MVA-labeled isoprenoid analyzed in the studies described above was in fact derived from proteins and not from a farnesol-bearing contaminant that might have remained with the precipitated protein material after the delipidation procedure, we repeated the CH$_3$I cleavage and chromatographic fractionations using proteins that were electroeluted from the 20-30 kDa region of SDS gels. Approxi- mately 95% of the $[^3]$H]MVA-derived radioactivity in the electroeluted protein was recovered in the chloroform after the cleavage reaction. As in the case of the total soluble protein (Table 1), 90% of this radioactive material was eluted in the chloroform fraction.
from a Sep-Pak C-18 column with methanol. Reverse-phase HPLC (Fig. 5A) yielded two peaks with retention times identical to those of peaks 1 and 2 in Fig. 2B. TLC again showed that peak 1 contained material comigrating with farnesol and nerolidol (Fig. 5B), whereas peak 2 contained the more hydrophobic material migrating near the solvent front (Fig. 5C). Thus, except for a higher proportion of radioactivity in peak 2, the radioactive material released by CHJ from the electroeluted 20-30-kDa proteins appeared to be essentially the same as that released from total delipidated soluble protein.

**DISCUSSION**

The foregoing chromatographic evidence suggests that farnesol is the principal isoprenoid involved in the post-translational modification of the 21-28-kDa mammalian cell proteins. The inability of the linkage to methyl iodide is consistent with a cysteine thioether bond to the isoprenoid group, similar to that found in the fungal mating factors (12-16). In the C-terminal modification of the 21-28-kDa mammalian cell protein (12, 13, 15, 16) and the isoprenoid modification appears to occur in conjunction with the intracellular processing of the peptides from larger precursors (22, 23). Recent evidence suggests that these features may be shared by the mammalian proteins which undergo farnesylation. For example, the post-translational processing of p21 involves several steps, including proteolytic removal of three C-terminal amino acids to expose a C-terminal cysteine (24), carboxymethylation of this cysteine (24, 25), and eventual acylation of a cysteine residue by palmitate (26, 27). In an elegant study, Hancock et al. (11) have demonstrated that isoprenoid modification of the C-terminal cysteine (Cys-186) in p21 precedes fatty acylation and is, therefore, essential for the membrane localization and transforming activity of the protein. Based on this finding, it is tempting to speculate that farnesylation may play a role in the C-terminal processing of other ras-related GTP-binding proteins (28, 29). In this regard, it is noteworthy that MEL cells do not synthesize p21 in amounts readily detectable by immunoblotting and that the 21-28-kDa [3H]MVA-labeled proteins in these cells are distinct from p21 on two-dimensional gels.2 Thus, it will be of great interest to determine whether or not these proteins represent novel members of the ras "superfamily.”

Recent immunologic evidence indicates that two nuclear matrix proteins (66-70 kDa) labeled with [3H]MVA correspond to the nuclear lamins A and B (5, 6). These proteins were not solubilized with Triton X-100 and hence were not represented in the present study. However, their predicted amino acid sequences have cysteine residues in the fourth position from the C-terminal (30, 31), and carboxymethylation of lamin B is known to influence its association with the nuclear envelope (22). Thus, the possibility that the [3H]MVA labeling of lamins A and B may be due to farnesylation of the cysteine nearest the C-terminal merits serious consideration. As mentioned earlier, most of the proteins modified by farnesol in mammalian cells remain to be identified. Determination of the structures of these proteins, the enzymatic mechanisms underlying the farnesylation, and the functional significance of this modification should prove to be important and challenging areas for future research.

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**REFERENCES**


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1 W. A. Maltese and K. M. Sheridan, unpublished data.