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PRENYLATED PROTEINS: DEMONSTRATION OF A THIOETHER LINKAGE TO CYSTEINE OF PROTEINS*

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SUMMARY: Prenylated amino acid fragments have been isolated from prenylated proteins of Chinese hamster ovary cells. Gel-exclusion chromatography indicates that these proteins are modified by two different prenyl groups. The prenyl-amino acid fragments are labeled by ^{35}S from cysteine, and this bond is cleaved by Raney-Ni, proving that the prenyl residue is linked to protein via a thioether to cysteine. Hydrazinolysis has been used to demonstrate that the cysteine is carboxy terminal.

Proteins have been shown to be modified by products of metabolism of mevalonate (MVA), the universal isoprenoid precursor (1,2,3). These proteins were discovered in cells in which endogenous MVA synthesis was blocked by inhibitors and fed radioisotopic MVA (1,4). In these and subsequent experiments, covalent linkage of MVA products to protein was indicated by susceptibility of the macromolecular material thus obtained to protease treatment as well as the electrophoretic mobility of the labeled products on polyacrylamide gel electrophoresis. Electrophoresis showed that only a few

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Abbreviations used in this paper: MVA, mevalonate; CHO, Chinese hamster ovary.

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proteins were labeled selectively (1,3,5). A specific protein, Lamin B, has been shown to be modified by a derivative of MVA (2,6,7). MVA-labeled peptides, isolated from these proteins, are nonpolar, suggesting that the MVA-derived material is a prenyl residue (2).

Important clues to the structure of prenylated proteins have come from the structure of fungal-mating factors. These peptides contain a farnesyl residue covalently attached to the sulfur of a carboxy terminal cysteine via an allylic thioether (8,9).

We have examined the incorporation of MVA into proteins of Chinese hamster ovary (CHO) K1 cells and now report some of the characteristics of the prenyl group and its link to protein. Several different isoprenoids have been found, which are covalently attached to protein via a thioether of cysteine, which is carboxy terminal.

MATERIALS AND METHODS

McCoy's 5A media and fetal bovine serum were purchased from Gibco Laboratories. Mevinolin was a gift of Dr. A. W. Alberts (Merck, Sharp & Dohme). Bio-Rad supplied Bio-Beads S-X4. Biochemicals were purchased from Sigma and other chemicals utilized were reagent grade. [5-^{3}H]-Mevalonic acid was purchased from NEN Research Products, and [^{35}S]-cysteine was from Amersham/Searle.

CHO-K1 cells (ATCC CCL 61) were grown in McCoy's 5A medium supplemented with 80 µg/ml gentamicin sulfate and 5% fetal bovine serum. Cultures were seeded at 1-2x 10^4 cells/cm and incubated at 37°C. in a 5% CO_2 atmosphere. After 2 or 3 days, media was removed and fresh media containing mevinolin (10 µg/ml), 25-hydroxycholesterol (1 µg/ml), cholesterol (4 µg/ml), α-tocopherol (4 µg/ml), and 2,(6)-di-tert-butyl-p-cresol (1 µg/ml) (10). Bovine serum albumin was included at a final concentration of 0.5% to aid in the solution of cholesterol and α-tocopherol. After one day's growth on this media, the volume of media was reduced to one-third and [5-^{3}H]-MVA, 135 Ci/mole, 6 µM, was added. In the experiments in which cells were labeled with [^{35}S]-cysteine, 2 µCi/ml of this amino acid were included in the media throughout.

Cells were collected by scraping them from the flasks and transferring them to conical centrifuge tubes. Cells were pelleted and rinsed by 3 successive portions of 0.9% NaCl containing 0.02% disodium EDTA. The cells were then extracted successively with three portions of absolute ethanol and then diethyl ether to remove lipids. The antioxidant, 2, 6 -di-tert-butyl-p-cresol (1 µg/ml) was included in all solvents. The cellular residue was heated at 100°C. in 1 ml of 14N KOH containing 20 mg pyrogallol under a stream of N_2. The prenyl fragment was transferred into 1-butanol by three successive extractions, and the combined butanol extracts were washed with H_2O. Butanol was removed by warming the solvent under a stream of N_2. The residue was acetylated by treatment with acetic anhydride in pyridine. The product was transferred into diethyl ether and methylated with diazomethane. Recovery of tritium from the cellular residue was quantitative.

Cleavage of the hydrolytic fragment was with Raney-Ni for 15 min at room temperature in methanol (11). Products were extracted into pentane.

Products were analyzed by chromatography on columns of Bio-Beads S-X4. The column, 1 x 84 cm, was calibrated with dolichol-19, cholesteryl linoleate, squalene, phytol, and farnesol. Toluene was the solvent.

For hydrazinolysis, dry, solvent extracted cellular residue was heated at 60°C for 20 h in hydrazine-containing hydrazine sulfate as a catalyst (12). The product was chromatographed on Amberlite CG-50 with alcohol-water as solvent.

RESULTS

For these experiments, CHO cells were grown to near confluence and then given mevinolin and 25-hydroxycholesterol, inhibitors of mevalonate biosynthesis. After one day of this treatment, the volume of media was reduced and cells were fed [^{3}H]MVA. After an additional day, cells were harvested and washed with saline. These cells incorporated about 1% of the administered isotope into cellular material. About a third of this radioactivity remained with the macromolecular residue after thorough extraction with ethanol and diethyl ether. While treatment of the cellular residue with either 6N $HClO_4$ or 1N KOH for 15 min at 100°C failed to release significant amounts of radioactivity that could be extracted into 1-butanol, heating at 95°C for 1 h in 14N KOH quantitatively converted the radioactivity to a 1-butanol-soluble form. Since this condition is sufficient for proteolysis, we conclude that the prenyl residue is covalently attached to protein.

The observation that the protein-to-prenyl linkage was stable suggested that proteolytic cleavage by strong base would yield the prenyl fragment covalently bound to an amino acid. The butanol-soluble material thus obtained was acetylated and methylated, which rendered the fragment toluene soluble. When the derivatized material was analyzed by chromatography on a column of Bio-Beads S-X4, the elution pattern showed two major peaks of radioactivity which bracketed the squalene molecular weight marker (Fig. 1, open circles).

The bond between the prenyl residue and the amino acid in the fragment was probed by treatment with Raney-Ni, a reagent that is specific for cleavage of sulfur-containing bonds (11). The hydrolytic fragment was reacted with Ni

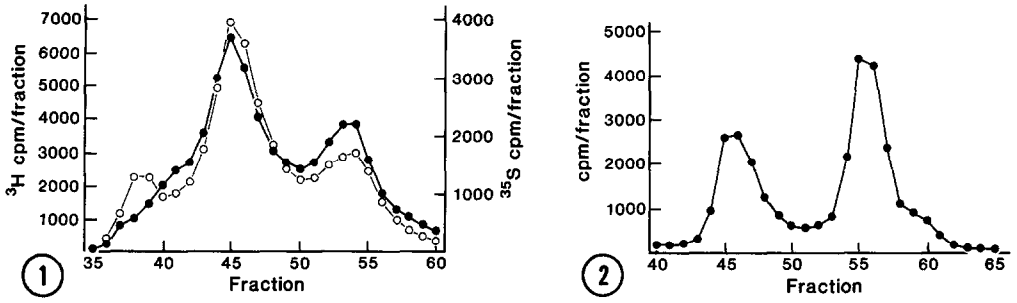

Figure 1. Chromatography on Bio-Beads S-X4 of hydrolytic fragments that had been derivatized. These fragments were isolated from CHO cells that were grown on [^{35}S]-cysteine-containing media and fed [5-^{3}H]-MVA. Open symbols are ^{3}H, closed ^{35}S. The column was 1 x 84 cm and 1.5-ml fractions were collected. Molecular weight standards eluted as follows: cholesteryl linoleate (M.W. 648) fractions 44-45; squalene (M.W. 410) fractions 48-49; phytol (M.W. 297) fractions 53-54; and farnesol (M.W. 222) fractions 61-62.

Figure 2. Chromatography on Bio-Beads S-X4 of the Raney-Ni cleavage products obtained from prenylated proteins. The column and elution position of standards are the same as Figure 1.

for a few minutes in alcohol at room temperature and the products extracted into pentane. Typically, half of the starting material was converted to pentane-soluble product. Gel-exclusion chromatography demonstrated the presence of two products whose elution pattern resembled that of the starting material (Fig. 2). However, these products were the result of cleavage of the bond to the amino acid, since they were soluble in nonpolar solvents and had markedly different patterns on thin-layer chromatography (data not shown). The sensitivity of the prenyl-to-amino acid bond to Raney-Ni demonstrates a thioether linkage between amino acid and prenyl residue. Cysteine is the only candidate for participating in such a bond, and this was proven by feeding [^{35}S]-cysteine to CHO cells. Cells were grown on media-supplemented [^{35}S]-cysteine and then labeled with [5-^{3}H]-MVA as before. The prenyl fragment obtained from these cells was derivatized and chromatographed on Bio-Beads S-X4. The elution profile demonstrates that both fragments were indeed labeled with sulfur, as well as tritium, and that the isotope ratio through the major

two peaks was nearly constant (Fig. 1). Since the prenyl residue of yeast-mating factor has been shown to be attached to a carboxy terminal cysteine (8), we subjected tritium-labeled CHO proteins to hydrazinolysis, a procedure which releases the carboxy terminal amino acid as a free amino acid. After hydrazinolysis, the radioactivity from the CHO proteins was recovered in the neutral amino acid fraction from the anion exchange resin. This indicates the prenylated cysteine is carboxy terminal.

DISCUSSION

In these experiments and those reported by others, the incorporation of isotope from MVA into macromolecular material has been relatively poor, making it possible that the labeling arises from a contaminant in the MVA or that the isotope had been converted to an alternate precursor via a shunt pathway. The use of [5-^{3}H]-MVA negates the latter consideration since this hydrogen is converted to a proton when MVA enters the shunt pathway (13). We also found, in other experiments, that [^{14}C]-MVA is a precursor of the prenylated fragments. These two observations make it clear that prenylated proteins are modified by a derivative of MVA. Our results, which show that the bond is stable to both acid and alkaline hydrolysis as well as the finding of ^{35}S incorporation from cysteine into the prenyl fragments, provide unequivocal evidence for a covalent bond between these entities. The fragments, which quantitatively account for protein prenylation in CHO cells, are labeled with sulfur from cysteine, and the sensitivity of this bond to cleavage with Ni shows that a thioether joins both prenyl residues to protein. Thus, mammalian-prenylated proteins have this structural feature in common with the yeast-mating factors (8,9). The results of hydrazinolysis demonstrate a second commonality in that the cysteine is carboxy terminal.

Analysis of the prenyl fragments has been initiated utilizing the hydrolytic fragments obtained from [^{3}H]-MVA-labeled proteins. While accurate estimation of the molecular weight of the hydrolysis products is not possible because they differ structurally from the standards used to calibrate the

column, the products obtained by cleavage of the prenylated fragment from the amino acid by Raney-Ni are suitable for such an analysis. The larger of the two products obtained by this procedure is estimated to have a molecular weight of between 400 and 700, while the smaller nearly migrated with a diterpene standard on gel-exclusion chromatography. This and other experiments (14) indicate that the prenylated CHO proteins contain a diterpene residue which is in contrast to the sesquiterpene found in yeast peptides. Thus, while there are strong similarities between prenylation of yeast and mammalian proteins, there are interesting differences that remain to be examined.

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