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Prenylated proteins: Synthesis of geranylgeranylcysteine and identification of this thioether amino acid as a component of proteins in CHO cells

(posttranslational modification/isoprenyl/mevalonate/polyisoprenyl)

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ABSTRACT **Prenylated proteins, labeled in the isoprenoid residue by growing CHO cells in medium containing [5-^{3}H]mevalonate, were degraded by three different proteolytic procedures, enzymatic or alkaline hydrolysis as well as hydrazinolysis. The products thus obtained were analyzed by HPLC with chemically prepared all-*trans*-geranylgeranylcysteine as a standard. About 10% of the radioactive products released by each lytic procedure showed the same chromatographic properties as geranylgeranylcysteine. This verifies the earlier conclusion, based on less-direct evidence, that this thioether derivative of cysteine is a component of naturally occurring proteins. The finding of this modified amino acid as a product of hydrazinolysis indicates that it is a carboxyl-terminal amino acid and that it is not carboxyl-methylated.**

Prenylation is a recently discovered posttranslational modification of proteins that directs cytosolic proteins to membranes and at the same time activates them functionally (1–3). The change in polarity essential for binding to membranes is accomplished by the covalent attachment of a polyisoprene to a carboxyl-terminal cysteine via a thioether bond. The prototype for prenylated proteins is a yeast mating pheromone, the a factor, which has farnesylcysteine as the carboxyl-terminal amino acid. In this peptide, the cysteine is also carboxyl-methylated (4). Similarities between the biological synthesis/processing of this pheromone and that of the oncogenic Ras proteins have led to substantial interest in the prenylation of proteins, and a number of reports indicate that farnesylation of proteins may be a general phenomenon (3, 5, 6). While some of the evidence that has been presented for the structure of the isoprenoid residue has been indirect, Farnsworth *et al.* (5) provided unequivocal data for the presence of a farnesyl group in lamin B. Enzymatic digests of this protein were treated with Raney nickel, a reagent that selectively cleaves carbon-sulfur bonds such as thioethers (7). A hydrocarbon fraction was obtained that, when analyzed by GC/MS, was found to be farnesene (5).

A homologous isoprenoid, a diterpene, has been isolated from prenylated proteins. A geranylgeranyl group has been shown to be a component of proteins from both HeLa (8) and CHO (9) cells. Enzymatic hydrolysis was used for proteins from HeLa cells, whereas peptide-bond cleavage of CHO proteins was accomplished with hydrazine. The cleavage fragments obtained by both procedures were treated with Raney nickel, and the resultant hydrocarbons were subjected to GC/MS analysis. The products were established to be diterpenes; i.e., geranylgeranyl-like.

The sensitivity of the bond between the isoprenoid and protein to Raney nickel indicates a sulfur linkage, and by implication cysteine should be the modified amino acid. Additionally, prenyl amino acid fractions have been labeled with ^{35}S from [^{35}S]cysteine, confirming the involvement of this amino acid (5, 10). We now report the synthesis of geranylgeranylcysteine and the direct identification of this amino acid derivative in cleavage fragments obtained from CHO proteins.

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MATERIALS AND METHODS

Unless otherwise noted, biochemicals were purchased from Sigma. Other chemicals used were reagent grade or the best available. [5-^{3}H]Mevalonate was a product of DuPont/NEN. CHO (Chinese hamster ovary) cells were grown, labeled with [5-^{3}H]mevalonate, and harvested as before (10). After washing with saline, suspensions of cells were sealed under argon and stored at −70°C. For analysis of prenyl fragments, cells equivalent to 1–4 mg of protein containing about 2×10^5 cpm of tritium were thawed and extracted three times with absolute ethanol containing 2,6-di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, BHT; 1 μg/ml) and then three times with diethyl ether.

Three methods were used for digesting the delipidated cellular residue. Hydrazinolysis was accomplished by dissolving the cellular residue in 0.2 ml of anhydrous hydrazine in a glass tube, which was then sealed under argon. After heating at 100°C for 16 hr, the hydrazine was removed *in vacuo*. For enzymatic digestion, cellular residue was suspended in 1 ml of 50 mM Na_2CO_3 containing 10 units of protease from *Streptomyces griseus* (Sigma type XXI). The incubation was at 31°C for 1 or 2 days. For the second day, either an additional 10 units of protease was added or 3 units of carboxypeptidase Y (Pierce), as well as protease, was included. Alkaline hydrolysis of the cellular residue was in 1 M KOH at 31°C for 1–4 days. Enzymatic and alkaline hydrolytic reactions were carried out in screw-capped vials in an atmosphere of argon. Pyrogallol was included to scavenge oxygen, and the lack of color change during incubation indicated successful exclusion of air. The prenyl-containing fragments were extracted into water-saturated 1-butanol containing butylated hydroxytoluene. The butanol was then washed once with water, once with 1 M KH_2PO_4, and finally three times with water. Butanol was removed with warming under a stream of nitrogen gas.

The radioactive products were dissolved in a small volume of methanol containing 1% trifluoroacetic acid and analyzed by HPLC with an Altex system using a Vydac C_{18} peptide/protein column (4.6 mm × 25 cm; 5-μm particle size). Elution was with a linear gradient from 60% acetonitrile/water, 1:1, in methanol to 100% methanol (both containing 0.1% trifluoroacetic acid) over 30 min followed by a 30-min isocratic elution with methanol containing 0.1% trifluoroacetic acid. The flow rate was maintained at 1.5 ml/min, and 1.05-ml fractions were collected. When geranylgeranylcysteine was

used as a marker, the effluent was monitored at 210 nm with a Gilson Holochrome monitor.

Geranylgeranylcysteine was synthesized from geranylgeranyl chloride and cysteine by a modification of the procedure of Armstrong and Lewis (11). The spectral data (IR, ^{1}H NMR, and ^{13}C NMR) were consistent with the assigned structure. High-resolution fast-atom bombardment MS m/z calculated for $C_{23}H_{40}NO_2S$, 394.2780; found, 394.2804.

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300 instrument. The IR spectra were obtained using a Nicolet PCIR system. The mass spectra were obtained using either a Finnigan MAT 731 or a VG Analytical 7060 instrument (Manchester, U.K.).

Geranylgeranyl chloride was prepared from geranylgeraniol by a modification of the method of Collington and Meyers (12). The spectral data (IR, ^{1}H NMR, and ^{13}C NMR) were consistent with the assigned structure. High-resolution chemical ionization MS m/z calculated for $C_{20}H_{33}Cl$, 308.2271; found, 308.2262.

RESULTS

The synthesis of geranylgeranyl chloride and geranylgeranylcysteine involved slight modifications of literature procedures and was routine. The identity of the products was verified by IR, NMR, and mass spectra.

Prenylated proteins were selectively labeled in the isoprene moiety by feeding [5-^{3}H]mevalonate to CHO cells that were blocked in the synthesis of this isoprenoid precursor (10). After the cells were extracted with organic solvents to remove lipids, total cellular proteins were proteolytically cleaved by three different methods. Enzymatic digestion of CHO cell protein quantitatively released the mevalonate label as a 1-butanol-soluble material, which was analyzed by HPLC. Five peaks of radioactivity were observed (Fig. 1). An important peak (fractions 15–17), which accounts for about 10% of recovered isotope, was eluted in the same fractions as authentic geranylgeranylcysteine (see Fig. 2 *Inset*). Routinely, 75–85% of the injected isotope was recovered on chromatography. Incubation with protease for 1 or 2 days, or inclusion of carboxypeptidase Y, changed neither the yield of butanol-soluble fragments or their distribution on HPLC.

In our earlier report on the structure of the prenyl entity, hydrazinolysis was used for protein cleavage (9), and this method is used here to establish geranylgeranylcysteine as a component of prenylated proteins. Labeled proteins were heated in a sealed tube at 100°C for 16 hr in anhydrous hydrazine with a small amount of geranylgeranylcysteine as carrier. After removal of hydrazine *in vacuo*, the residue was dissolved in methanol containing trifluoroacetic acid and analyzed by HPLC. The elution pattern showed coincidence of a peak of radioactivity with the mass of geranylgeranylcysteine (Fig. 2). About 20% of recovered isotope was associated with this peak.

Strong base also can be used for hydrolysis of peptide bonds. Samples of delipidated CHO cells were dispersed in 14 M KOH and held at 31°C for 1–4 days. The mevalonate-labeled material could be quantitatively extracted into 1-butanol after this treatment. On HPLC, a principal band of radioactivity was found and this band was at the elution position of geranylgeranylcysteine (Fig. 3). The elution pattern of the alkaline hydrolysis products was different from that obtained with the other lytic procedures in that only one band was found and there was a high baseline of radioactivity throughout the elution of the column. About 60% of injected isotope was recovered on chromatography of the alkaline hydrolysis products. Of that, 15–20% was associated with geranylgeranylcysteine.

DISCUSSION

Prenylated CHO proteins were subjected to three different proteolytic procedures. The three methods yielded geranylgeranylcysteine in comparable amounts, i.e., about 10% of the isotope released on protein cleavage. Thus, recovery of this material is comparable to that reported previously (9). In those experiments the yield of prenylcysteine was almost 50% on hydrazinolysis. Treatment with Raney nickel, which was used to desulfurize this product, gave hydrocarbons in 30% yield. Thus, overall recovery of the geranylgeranyl fragment for MS analysis was almost 15%. Farnesylcysteine, a component of other prenylated proteins, would be eluted in the early portion of the HPLC program used. With alkaline as well as enzymatic hydrolysis, the yield of geranylgeranylcysteine was constant for several times of incubation (data not shown). The low but constant yield of geranylgeranyl-

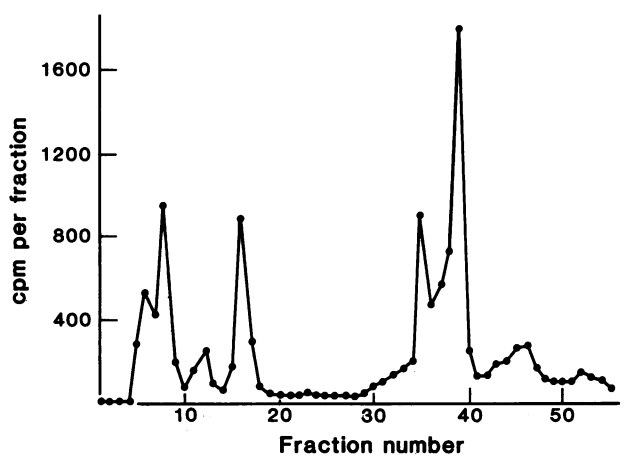

FIG. 1. HPLC of the products of proteolytic digestion of prenylated CHO proteins. Of the radioactive material applied to the column, 72% was recovered, and 11% of that recovered was found in fractions 15–17, the elution volume of geranylgeranylcysteine.

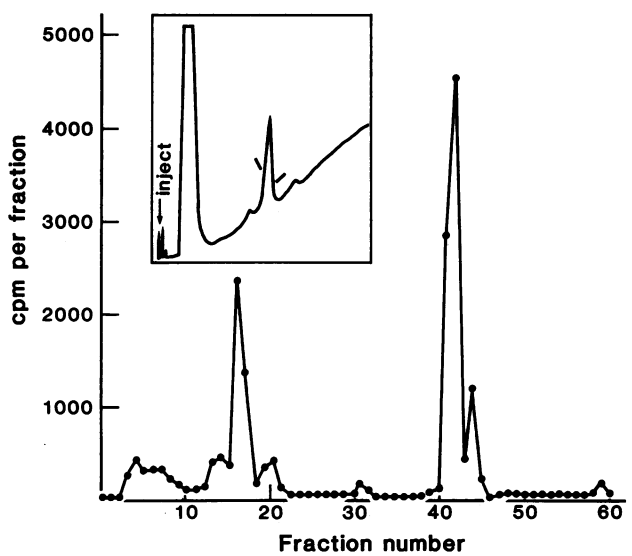

FIG. 2. HPLC of hydrazinolysis products from prenylated CHO proteins. *Inset* is a recording of absorbancy (210 nm) of the effluent; the tick marks represent the initiation and termination of collection of fractions 17 and 18. The tube between the detector and fraction collector had a volume of 0.55 ml, which accounts for the apparent half-fraction offset between A_{210} and radioisotope. Of the radioactive material applied to the column, 50% was recovered, and 19% of that was found in fractions 17 and 18.

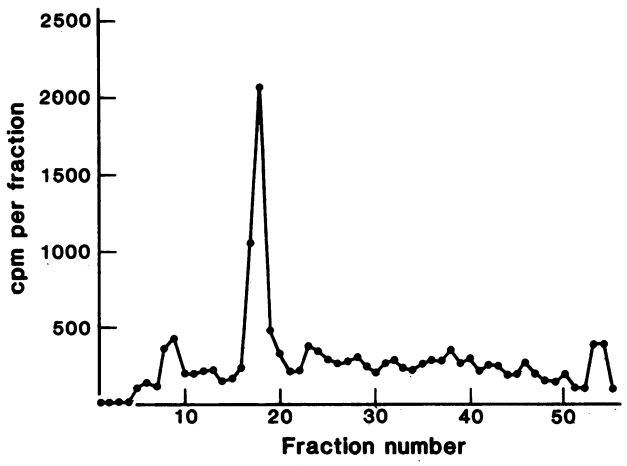

FIG. 3. HPLC of alkaline hydrolysis (20 hr, 14 M KOH) products from prenylated CHO proteins. Of the material applied to the column, 61% was recovered, and 16% of that was in fractions 17 and 18.

cysteine irrespective of the method of hydrolysis, as well as time of hydrolysis, indicates that other prenylated components may be present. In earlier experiments, we found that strong alkaline hydrolysis released two different prenyl fragments that were equivalently labeled by ^{3}H from mevalonate and ^{35}S from cysteine (10). By this criterion, all of the labeled material recovered by butanol extraction was from prenylated proteins. This consideration suggests that the other peaks of radioactivity observed on HPLC of the proteolytic products represent other prenyl entities. The finding of a continuous high base line of radioactivity on chromatography of the products of alkaline hydrolysis suggests that these materials may be alkali-labile.

The recovery of geranylgeranylcysteine on hydrazinolysis is especially significant. In the yeast mating factor **a**, the carboxyl group of the prenyl-bearing cysteine is methylated. During hydrazinolysis, this methyl group would be displaced by hydrazine, yielding a hydrazide that would be stable under the conditions we used for isolation and chromatography of products. Hydrazides are polar and would be eluted early on the chromatogram. We found, however, that hydrazinolysis gave the best yields of geranylgeranylcysteine, and relatively little polar material was recovered during chromatography. These observations are consistent with data presented earlier, which show that, for CHO cells, relatively little, if any, of the prenylated cysteine is carboxyl-methylated (10). This is in contrast to farnesylated proteins, which apparently are methylated. The methylation of the carboxyl-terminal cysteine of farnesylated proteins in contrast to proteins modified by a geranylgeranylresidue may be of significance. The function of prenylation is to target selected proteins to membranes, and there is a significant difference in the contiguous hydrocarbon chain length in these two isoprenoids, 16 vs. 12 carbons. The longer chain length closely matches the chain length of the fatty acids most commonly found in membranes. A rationale for methylation of the carboxyl of farnesylated cysteine would be the neutralization of the charge of this group, thereby decreasing polarity and increasing its potential to partition into the membrane bilayer.

Our results demonstrate that geranylgeranylcysteine is a component amino acid of prenylated CHO proteins. The demonstration of this modified amino acid in proteolytic, as well as chemical, digests indicates that it is not an artifact of the condition of protein cleavage. In addition, we have verified earlier conclusions that were based on products obtained by cleavage with Raney nickel (8, 9). This prenylated amino acid is at the carboxyl terminus and is not methylated. Our data also suggest that other prenyl entities may be present.

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