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UTILIZATION OF GERANYLGERANIOL FOR PROTEIN ISOPRENYLATION IN C6 GLIAL CELLS[1]

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Summary: When rat C6 glial cells were incubated with [^{3}H]geranylgeraniol (**GGol**), radioactivity was incorporated into a delipidated protein fraction. SDS-PAGE analysis of the protein fraction labeled by [^{3}H]GGol revealed a 46 kDa polypeptide and a group of labeled polypeptides (19-27 kDa) in the same size range as the small GTP-binding proteins. A similar pattern of labeled polypeptides was seen when C6 cells were metabolically labeled with [^{14}C]mevalonolactone. An isotopically labeled product, chromatographically identical to geranylgeranyl-cysteine (**GG-Cys**), was released by Pronase E digestion of the [^{3}H]GGol-labeled protein. When the protein fraction from cells metabolically labeled with [^{3}H]mevalonolactone was digested with Pronase E, two radiolabeled products were released with the chromatographic mobilities of farnesyl-cysteine (**F-Cys**) and GG-Cys. These studies suggest that C6 glial cells are capable of converting GGol to geranylgeranyl pyrophosphate (**GG-P-P**), or perhaps a novel "activated" form of the allylic isoprenol, that can be utilized for protein isoprenylation reactions.

A diverse set of cellular proteins in eukaryotes have recently been shown to contain hydrophobic prenyl groups, either farnesyl (C15) or geranylgeranyl (C20), attached by thioether linkages to cysteinyl residues at or near the C-terminus (1,2). In mammals, estimates suggest that isoprenoid-modified proteins comprise 0.5-2% of total cellular proteins with the majority being modified by one or more geranyl-geranyl units (3). The list of geranylgeranylated proteins is extensive. It includes

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Abbreviations: Cys, cysteine; X, unspecified amino acid; GG-P-P, geranylgeranyl pyrophosphate; GGol, geranylgeraniol; F-P-P, farnesyl pyrophosphate; GG-Cys, geranylgeranyl-cysteine; F-Cys, farnesyl-cysteine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CoQ, ubiquinone (coenzyme Q).

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small GTP-binding proteins of the Rho and Rac families involved in the control of cytoskeletal-membrane interactions (4, 5) as well as the Rab family implicated in the regulation of vesicular fusion during secretion and endocytosis (2,6-8). These proteins are initially translated with C-terminal Cys-X-X-X, Cys-X-Cys or Cys-Cys sequences which are recognized by geranylgeranyltransferases which utilize geranylgeranyl pyrophosphate (GG-P-P) as the "activated" isoprenyl donor.

In this study we have found that rat C6 glial cells can utilize the free allylic isoprenol, geranylgeraniol (GGol), as a metabolic source of the isoprenyl chain for protein isoprenylation. The majority of the proteins metabolically labeled by [^{3}H]GGol are in the size range of small G proteins. These findings suggest that brain and other mammalian cells, have the enzymatic capacity to reform GG-P-P, and possibly farnesyl pyrophosphate (F-P-P), from the allylic isoprenols.

MATERIALS AND METHODS

Materials: ω,t,t,t-[1-^{3}H]Geranylgeraniol (57 Ci/mmol) and RS-[5-^{3}H(N)]mevalonolactone (50-60 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). RS-[2-^{14}C]mevalonolactone (50.1 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Geranylgeraniol was a generous gift from Dr. M. Mizuno of Kuraray (Okayama, Japan). Mevinolin was provided by Dr. A. Alberts (Merck Sharp & Dohme Res. Lab, Division of Merck & Co., Inc., Rahway, NJ). Geranylgeranyl-cysteine (GG-Cys) and farnesyl-cysteine (F-Cys) were synthesized as described by Kamiya et al. (9) and purified by preparative TLC. Ubiquinone (CoQ), antibiotic-antimycotic lyophylized powder, heat-inactivated fetal bovine serum (FBS) and protease XIV from *Streptomyces griseus* (Pronase E) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's phosphate-buffered saline (PBS), Ham's F-10 medium, and sodium bicarbonate were purchased from GIBCO (Grand Island, NY). All other solvents and chemicals were reagent grade and purchased from standard commercial sources.

Cell culture: ATCC CCL 107 C6 glial cells (originally cloned from a rat glial tumor) obtained from American Type Culture Collection (Rockville, MD) were maintained in Ham's F-10 medium containing 1.2 g/L sodium bicarbonate, 10% FBS and antibiotic/antimycotic mixture. All cultures were incubated at 37°C in an atmosphere of 5 % CO_2 in air and grown to near confluence in Falcon 3001 tissue culture plates.

Metabolic labeling experiments: For metabolic labeling experiments with [^{3}H]GGol as the isotopic precursor, the labeled isoprenol was dissolved in ethanol and added to a small conical tube. The solvent was evaporated under a stream of nitrogen, and 1 µl FBS/µCi of [^{3}H]GGol was added. The mixture was placed in a Branson bath sonicator for 5 min. After sonication an aliquot was taken to verify that all of the [^{3}H]GGol had been dispersed in the serum. After removal of medium containing antibiotics, antibiotic-free medium (500 µl) and FBS (final concentration = 3%) containing [^{3}H]GGol was added to cell cultures (2-8 X 10^5 cells/dish) and incubated at 37 °C under 5% CO_2 for the indicated period of time. The labeled cells

were washed with ice-cold PBS, scraped from the dishes and transferred to a conical tube. The cell suspension was centrifuged (200 X g, 5 min), and the PBS removed by aspiration. CH_3OH (2 ml) was added, and the suspension was sonicated in a bath sonicator to disrupt the cell pellet. The mixture was centrifuged to sediment the delipidated protein, and the CH_3OH extract was transferred to another conical tube. The protein pellet was re-extracted twice with 2 ml of $CHCl_3/CH_3OH$ (2:1), and the lipid extracts were pooled. The organic solvent was evaporated under a stream of nitrogen, and the lipid residue redissolved in $CHCl_3/CH_3OH$ (2:1) or $CHCl_3/CH_3OH/H_2O$ (10:10:3). An aliquot was taken to determine the amount of radioactivity incorporated into lipid. Authentic geranylgeraniol, cholesterol, dolichol and CoQ standards were added to the lipid extracts, and they were analyzed on Merck silica gel G 60 TLC plates (Sigma, St. Louis, MO) by developing with hexane/diethyl ether/acetic acid (65:35:1). Standard compounds were located with iodine vapor or an anisaldehyde spray reagent (10), and radioactive zones were located with a Bioscan Imaging Scanner System 200-IBM.

After the residual organic solvent was removed by evaporation under a stream of nitrogen, the delipidated protein fractions were dissolved in 2% SDS-5 mM β-mercaptoethanol. An aliquot was used to determine the amount of labeled precursor incorporated into protein, and the remaining sample was analyzed by SDS-PAGE .

For metabolic labeling experiments with radiolabeled mevalonolactone, the medium containing antibiotics was removed by aspiration and 20-60 μCi of the radiolabeled precursor was added in 520 μl of antibiotic-free medium containing 3% FBS and 5 μg/ml of mevinolin. Cell cultures were incubated at 37°C for 18 h, and the labeled products were assayed as described above.

Pronase E digestion of the labeled protein fraction and chromatographic analysis of the labeled products: Delipidated protein fractions (50-100 μg) from C6 glial cells metabolically labeled with [^{3}H or ^{14}C] mevalonolactone or [^{3}H]GGol were resuspended in 100 μl 50 mM HEPES (pH 7.4)-2 mM calcium acetate in a bath sonicator. The labeled proteins were incubated with Pronase E (2 mg) at 37°C for 18 h. Proteolysis was terminated by the addition of 1 ml of n-butanol saturated with H_2O, and the mixture was centrifuged at 1,500 X g for 5 min to produce two phases. An aliquot of the n-butanol phase (upper), containing [^{3}H or ^{14}C]isoprenylated cysteines, was taken to determine the amount of radioactivity present. The n-butanol was evaporated under a stream of nitrogen, and the labeled products were redissolved in $CHCl_3/CH_3OH/H_2O$ (10:10:3) containing authentic F-Cys and GG-Cys. The butanol-soluble products were analyzed chromatographically on silica gel G 60 TLC plates developed with $CHCl_3/CH_3OH$ /7 N NH_4OH (45:50:5), or on Si*C_{18} reverse-phase TLC plates (J. T. Baker Inc., Phillipsburg, NJ) developed with acetonitrile/H_2O/acetic acid (75:25:1). Standard compounds were located with iodine vapor and/or an anisaldehyde spray reagent (10), and radioactive zones were located with a Bioscan Imaging Scanner System 200-IBM.

RESULTS AND DISCUSSION

Incorporation of [^{3}H]Geranylgeraniol into Proteins in C6 Glial Cells: When C6 glial cells were incubated in the presence of [^{3}H]GGol, radioactivity was incorporated into the delipidated protein fraction. The amount of radiolabeled

protein formed increased steadily over a 36 h time course (Figure 1) and was linearly dependent on the concentration of [^{3}H]GGol added to the incubation medium (Figure 2). The amount of radioactivity incorporated into the protein fraction was not affected by the addition of mevinolin (5 μg/ml), a concentration that reduces the incorporation of [^{3}H]acetate into cholesterol by at least 97%.

Surprisingly, under the same culture conditions little or no metabolically labeled CoQ was detected (data not shown). The incorporation of the isotopic precursor into CoQ would be expected if [^{3}H]GGol was converted to all-trans-[^{3}H]GG-P-P (11). It is possible that [^{3}H]GGol is incorporated into protein via [^{3}H]GG-P-P synthesized and utilized in a compartment that is inaccessible to the enzymes involved in CoQ biosynthesis.

SDS-PAGE Analysis of the Metabolically Labeled Proteins: To examine the size and number of the polypeptides labeled by incubating C6 glial cells with [^{3}H]GGol, the delipidated protein fractions were analyzed by SDS-PAGE. From Figure 3 (left lane), it can be seen that several radiolabeled polypeptides in the range 19-29 kDa, similar to the size of small G proteins (2,6) were metabolically labeled. Radioactivity was also incorporated into another apparently geranyl-geranylated 46 kDa polypeptide.

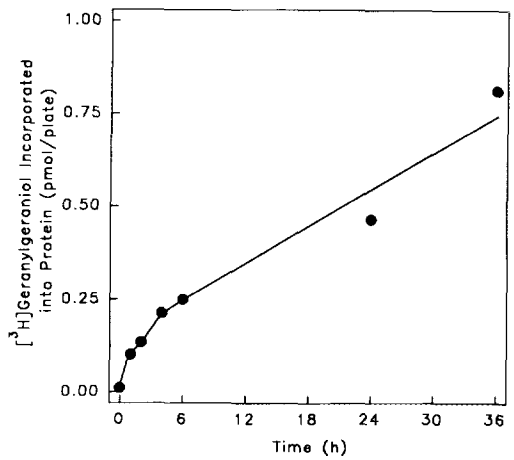

Figure 1. Time course for the incorporation of [^{3}H]geranylgeraniol into cellular proteins in C6 glial cells. Cells were incubated in medium containing 225 nM [^{3}H]geranylgeraniol (63 cpm/nmol) for the indicated time. The incorporation of the isotopic precursor into the protein fraction was assayed as described in **Materials and Methods**.

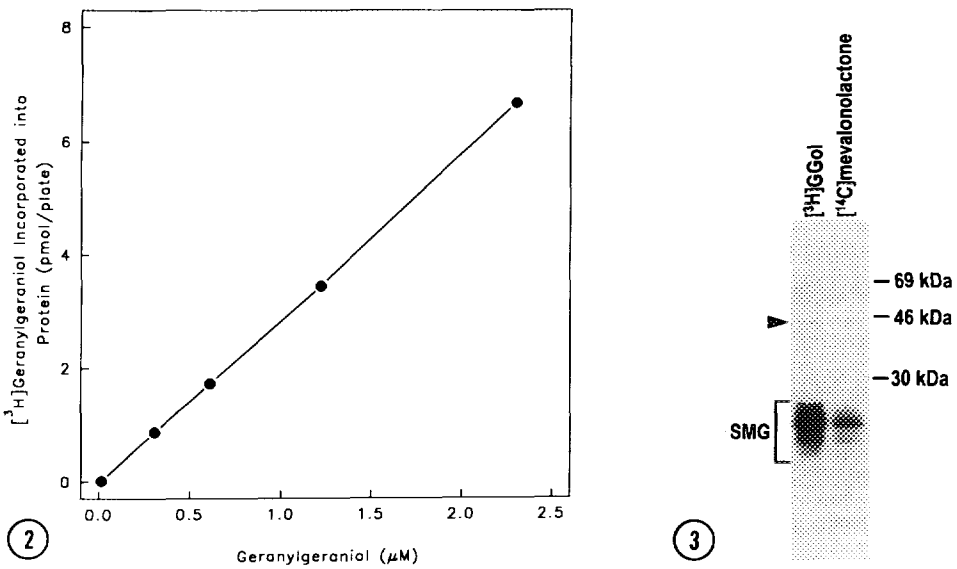

Figure 2. Dependence of labeled proteins formed on the concentration of [^{3}H]geranylgeraniol added to the culture medium. The concentration of the isotopic precursor was varied as indicated, and the amount of label incorporated into the protein fraction during a 18 h incubation period at 37 °C was assayed as described in **Materials and Methods**.

Figure 3. SDS-PAGE analysis of proteins labeled by incubating C6 glial cells with [^{3}H]geranylgeraniol (left lane) or [^{14}C]mevalonolactone (right lane). The delipidated protein fractions were analyzed by SDS-PAGE using a 12% polyacrylamide resolving gel and a 4% stacking gel (15). The gel was treated with ENTENSIFY (Dupont-NEN, Boston, MA) dried and exposed to X-ray film for 90 h. The calibration markers are indicated on the right side of the gel. The designation SMG refers to the range of molecular weights of small (19-27 kDa) G proteins, most of which are geranylgeranylated (2,3,6,15). The arrowhead indicates the position of a newly identified protein which appears to be geranylgeranylated.

When cells were incubated with [^{14}C]mevalonolactone under identical conditions (Figure 3, right lane), labeled polypeptides corresponding to the bands labeled by incubation with [^{3}H]GGol were seen in addition to several other, presumably [^{14}C]farnesylated polypeptides (15).

Release of [^{3}H]Geranylgeranyl-Cysteine by Pronase E Digestion of Radiolabeled Protein Fraction: As a preliminary attempt to determine if [^{3}H]GGol was covalently attached to cysteine residues, the radiolabeled protein fractions were digested with Pronase E , and the protease digestion products were analyzed by normal and reverse-phase chromatography. Protease-treatment of the labeled protein fraction released 70-80% of the radioactivity in a butanol-soluble form. When the butanol-soluble products were analyzed, a major labeled peak with the

mobility of GG-Cys on TLC plates developed with normal-phase (Figure 4, panel A) and reverse-phase chromatography systems (Figure 4, panel B) was observed. No labeled F-Cys was detected in protein fractions metabolically labeled *via* [^{3}H]GGol. Another significant peak of lower mobility was seen in both systems which could be an incompletely digested isoprenylated peptide or a multiply geranylgeranylated peptide containing more than one cysteinyl residue.

When the butanol-soluble products released by protease treatment of protein fractions metabolically labeled with [^{3}H or ^{14}C]mevalonolactone were analyzed, two major labeled products were detected with the same mobilities as F-Cys and GG-Cys (Figure 4, panels C and D). The labeled product of lower mobility noted above, was also observed.

These preliminary studies with C6 glial cells strongly suggest that free GGol can be utilized for protein isoprenylation. If GGol is enzymatically converted to

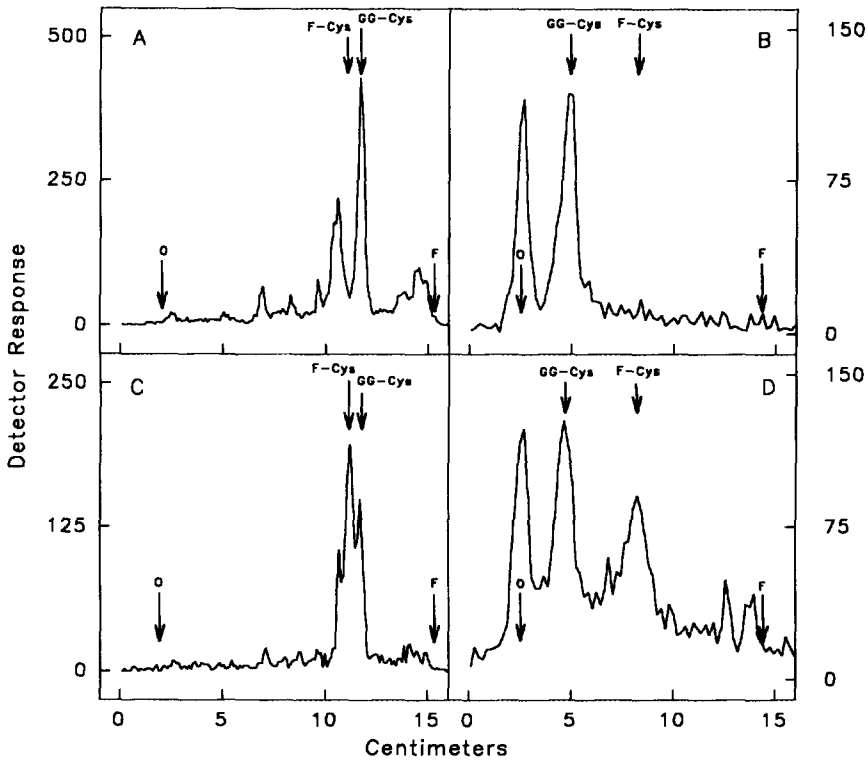

Figure 4. Chromatographic analysis of butanol-soluble products formed by Pronase E digestion of proteins metabolically labeled by incubation with [^{3}H]geranylgeraniol (panels A and B) or [^{3}H]mevalonolactone (panel C) or [^{14}C]mevalonolactone (panel D). The labeled products were analyzed by normal-phase (panels A and C) and reverse-phase (panels B and D) chromatography systems as described in **Materials and Methods**. O and F denote positions of origin and solvent front, respectively.

GG-P-P, the conventional isoprenyl donor for protein:geranylgeranyltransferases, brain cells and possibly other mammalian cells, may contain a novel enzyme system that converts the free allylic isoprenol to GG-P-P. If these enzymes exist, it will be interesting to see if they are related to the CTP-mediated kinase that phosphorylates the long chain polyisoprenol, dolichol (12-14). The "reactivation" of the free allylic isoprenyl unit would provide a mechanism for salvaging GGol produced by metabolic turnover of the isoprenylated proteins.

Studies are in progress to evaluate the utilization of free GGol and farnesol for protein isoprenylation in other mammalian cells, and to determine if the free allylic alcohols are enzymatically converted to GG-P-P and F-P-P or previously uncharacterized "activated" isoprenyl donors.

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