Journal of Neurochemistry
Lippincott-Raven Publishers, Philadelphia

Geranylgeraniol Overcomes the Block of Cell Proliferation by Lovastatin in C6 Glioma Cells

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Abstract: It is well documented that 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors prevent cultured mammalian cells from progressing through the cell cycle, suggesting a critical role for a mevalonate-derived product. Recently, it has been shown that free geranylgeraniol (GG-OH) and farnesol (F-OH) can be utilized by C6 glioma cells for protein isoprenylation. The ability of GG-OH and F-OH to restore protein geranylgeranylation or farnesylation selectively has enabled us to examine the possibility that mevalonate is essential for cell proliferation because it is a precursor of farnesyl pyrophosphate or geranylgeranyl pyrophosphate, the isoprenyl donors involved in the post-translational modification of key regulatory proteins. In this study we report that GG-OH, as well as mevalonate, overcomes the arrest of cell proliferation of C6 glioma cells treated with lovastatin, as assessed by increased cell numbers and a stimulation in [^{3}H]thymidine incorporation. The increase in cell number and [^{3}H]thymidine incorporation were significantly lower when F-OH was added. Under these conditions [^{3}H]mevalonate and [^{3}H]GG-OH are actively incorporated into a set of isoprenylated proteins in the size range of small, GTP-binding proteins (19–27 kDa) and a polypeptide with the molecular size (46 kDa) of the smaller isoform of 2′,3′-cyclic nucleotide 3′-phosphodiesterase. Analysis of the proteins metabolically labeled by [^{3}H]mevalonate and [^{3}H]GG-OH reveals the presence of labeled proteins containing geranylgeranylated cysteinyl residues. Consistent with geranylgeranylated proteins playing a critical role in the entry of C6 cells into the cell cycle, a (phosphonoacetamido)oxy derivative of GG-OH, a drug previously shown to interfere with protein geranylgeranylation, prevented the increase in cell number when mevalonate or GG-OH was added to lovastatin-treated cells. These results strongly suggest that geranylgeranylated proteins are essential for progression of C6 cells into the S phase of the cell cycle and provide the first evidence that the "salvage" pathway for the utilization of the free isoprenols is physiologically significant in the CNS. **Key Words:** Lovastatin—Geranylgeraniol—Geranylgeranyl pyrophosphate—Protein isoprenylation—Cell cycle.
J. Neurochem. **70,** 2397–2405 (1998).

Studies performed with 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors nearly 20 years ago firmly established that a mevalonate-derived product is involved in the progression of mammalian cells through the cell cycle (Quesney-Huneeus et al., 1979; Habenicht et al., 1980; Fairbanks et al., 1984). Pardee and co-workers (Keyomarsi et al., 1991) later established that lovastatin could be used to synchronize cultured cells in the G_1 phase. Related studies by Volpe and co-workers also demonstrated that mevalonate biosynthesis was required for the proliferation and differentiation of glial cells (Volpe and Obert, 1983; Bhat and Volpe, 1984; Volpe et al., 1985; Langan and Volpe, 1986, 1987). Because of the regulatory implications of these observations, there has been considerable interest in identifying the precise product formed via mevalonate that is required for cell growth.

HMG-CoA reductase, the enzyme potently inhibited by lovastatin (Alberts et al., 1980), catalyzes the formation of mevalonate, which is an important rate-controlling step in the biosynthesis of cholesterol (Goldstein and Brown, 1990). Farnesyl pyrophosphate (F-P-P), a mevalonate-derived intermediate, is formed at a key branch point in isoprenoid biosynthesis (Fig. 1). F-P-P is utilized for the biosynthesis of many compounds, including cholesterol, dolichyl phosphate, ubiquinone, heme A, and isoprenylated proteins (Grunler et al., 1994). Thus, any of these branches of the isoprenoid biosynthetic pathway potentially could have produced the critical mevalonate-derived product(s)

Received October 1, 1997; revised manuscript received December 22, 1997; accepted January 8, 1998.

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Abbreviations used: CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; F-Cys, farnesylcysteine; F-OH, farnesol; F-P-P, farnesyl pyrophosphate; GG-Cys, geranylgeranylcysteine; GG-OH, geranylgeraniol; GG-P-P, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PAG, a (phosphonoacetamido)oxy derivative of geranylgeraniol; PBS, phosphate-buffered saline; SS, Serum Supreme.

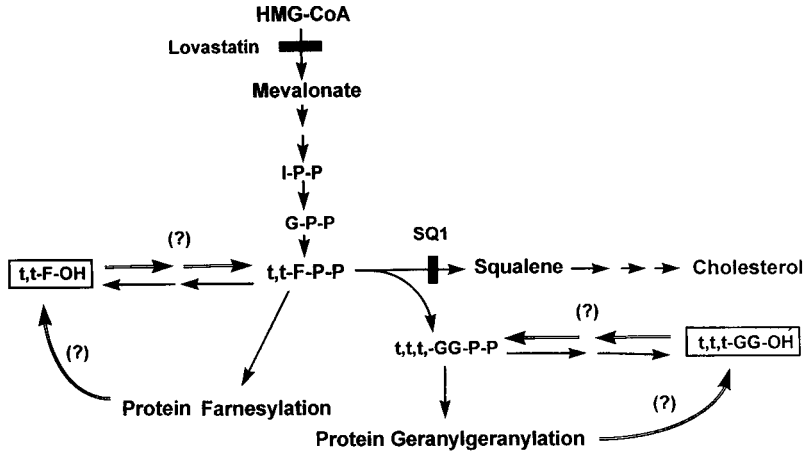

FIG. 1. Proposed salvage pathways for restoring protein farnesylation or geranylgeranylation selectively by exogenously supplied F-OH and GG-OH in lovastatin-treated C6 glioma cells. Question marks indicate enzymatic reactions that have not been extensively characterized. SQ, squalestatin 1; I-P-P, isopentenyl pyrophosphate.

involved in cell cycle control. However, supplementation experiments eliminated most of the candidates listed above as the required mevalonate-derived molecule(s) (Schmidt et al., 1982; Quesney-Huneeus et al., 1983; Fairbanks et al., 1984; Maltese and Sheridan, 1985; Langan and Volpe, 1987; Goldstein and Brown, 1990; Chakrabarti and Engleman, 1991).

An early series of studies in which cellular proteins were metabolically labeled by radiolabeled mevalonate (Schmidt et al., 1984; Sinensky and Logel, 1985; Maltese and Sheridan, 1987; Sepp-Lorenzino et al., 1991) raised the possibility that the mevalonate requirement for cell growth could be related to the isoprenylation of proteins. Since then, substantial progress has been made in the characterization of the isoprenylated cysteine residues, the thioether linkage, and the protein:isoprenyltransferases catalyzing this posttranslational modification, and it is now clear that several regulatory proteins, including lamin B, $p21^{ras}$, and most members of the small GTP-binding protein superfamily, are modified posttranslationally by isoprenylation (for reviews of the development of this field, see Maltese, 1990; Clarke, 1992; Schafer and Rine, 1992; Casey, 1994; Zhang and Casey, 1996). This information and the direct evidence that isoprenylation of $p21^{ras}$ is blocked by lovastatin treatment of COS cells (Hancock et al., 1989) and Chinese hamster ovary-K1 cells (Leonard et al., 1990) are consistent with the idea that the arrest of cell growth by HMG-CoA reductase inhibitors could be due to protein isoprenylation being impaired by the depletion of the F-P-P and geranylgeranyl pyrophosphate (GG-P-P) pools.

The utilization of free farnesol (F-OH) for isoprenoid biosynthesis was first demonstrated in the colonial microalga *Botryococcus braunii* (Huang and Poulter, 1989). Work in this laboratory has shown that when C6 glial cells are incubated with exogenous *trans, trans,trans*-[^{3}H]geranylgeraniol (GG-OH), a group of proteins in the size range of small-molecular-weight GTP-binding proteins and a polypeptide with an apparent molecular weight similar to the smaller isoform of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNPase) are metabolically labeled (Crick et al., 1994). Characterization of the products released by digestion of the metabolically labeled proteins with Pronase E indicated that the C6 proteins contain geranylgeranylated cysteine residues. Similarly, when rat C6 glial cells were incubated with [^{3}H]F-OH, radioactivity was incorporated into proteins containing farnesylated cysteine residues and into sterol by a squalestatin-sensitive mechanism (Crick et al., 1995*a*). In related studies Fliesler and Keller (1995) have shown that cholesterol was metabolically labeled when [^{3}H]F-OH was injected intravitreally into rats. For a recent review on the current understanding of the salvage pathway for the utilization of F-OH and GG-OH, see Crick et al. (1997*b*).

The incorporation of F-OH and GG-OH into isoprenylated proteins provides evidence for an alternate mechanism for the biosynthesis of F-P-P and GG-P-P. We have taken advantage of the ability of exogenous F-OH and GG-OH to restore protein farnesylation or geranylgeranylation selectively (Fig. 1) to determine if cell growth was arrested in lovastatin-treated C6 cells owing to depletion of the F-P-P and/or GG-P-P pools, consequently preventing the isoprenylation of regulatory proteins playing essential roles in cell cycle progression. All of the results are consistent with one or more geranylgeranylated proteins being involved in regulating cell proliferation in C6 glioma cells and provide evidence that the conversion of GG-OH to GG-P-P, or perhaps another novel isoprenyl donor, is a physiologically significant mechanism in the CNS. Some aspects of this work have been reported in preliminary form (Crick et al., 1996).

MATERIALS AND METHODS

Materials

ω,trans,trans-[1-^{3}H]F-OH (20 Ci/mmol), *ω,trans,trans, trans*-[1-^{3}H]GG-OH (57 Ci/mmol), *RS*-[5-^{3}H(N)]-mevalonolactone (60 Ci/mmol), and [*methyl*-^{3}H]thymidine (69 Ci/mmol) were purchased from American Radiolabeled

Chemicals (St. Louis, MO, U.S.A.). GG-OH and F-OH were generous gifts from Dr. M. Mizuno of Kuraray (Okayama, Japan). Protease XIV from *Streptomyces griseus* (Pronase E) was obtained from Sigma Chemical Co. (St. Louis). Dulbecco's phosphate-buffered saline (PBS), Ham's F-10 medium, trypsin (0.25%)–1 m*M* EDTA, and sodium bicarbonate were purchased from GibcoBRL (Grand Island, NY, U.S.A.). Serum Supreme (SS) was obtained from BioWhittaker (Walkersville, MD, U.S.A.). Baker SI*C18 reverse phase-octadecyl TLC plates were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). The dipotassium salt of (*trans, trans,trans*)-[2-oxo-2-[[3,7,11,15-tetramethyl-(2,6,10,14-hexadecatetraenyl)oxy]amino]ethyl]phosphonic acid, which is a (phosphonoacetamido)oxy derivative of GG-OH (PAG), was prepared and purified as described by Macchia et al. (1996). All other solvents and chemicals were reagent grade and were purchased from standard commercial sources.

Cell culture

The C6 glioma cells were purchased from ATCC (Rockville, MD, U.S.A.). Cells were maintained in Ham's F-10 medium containing 5% SS (complete medium). All cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air.

Proliferation assays

C6 cells were grown to confluence in 75-cm^2 tissue culture flasks in complete medium. The cells were detached from the flask with trypsin-EDTA (30 s, 23°C) and resuspended in complete medium. Cells (5,000–20,000 per well) were added to the wells in Linbro 24-well tissue culture dishes in 1 ml of complete medium and allowed to attach overnight. The medium was aspirated and replaced with Ham's F-10 containing 5% SS, lovastatin (5 μg/ml), and the indicated concentration of mevalonolactone or isoprenol. To add F-OH and GG-OH to the growth medium, the isoprenols were dissolved in chloroform/methanol (2:1 vol/vol) and added to a small conical tube. The solvent was evaporated under a stream of air, and the isoprenols were suspended in SS by sonication in a Branson bath sonicator for 10 min. This procedure quantitatively dispersed the amounts of isoprenols used in these studies. After the indicated interval the medium was removed by aspiration, and the cells were washed with PBS. PBS containing 2 m*M* EDTA was then added to the wells and left at room temperature for 5 min or until the cells detached from the dishes. The viable cells were then counted in a hemocytometer. Based on trypan blue exclusion, at least 95% of the cells were viable in the mevalonate- and GG-OH-supplemented cultures.

Metabolic labeling of C6 cells

For metabolic labeling experiments using [^{3}H]F-OH, [^{3}H]GG-OH, or [^{3}H]mevalonolactone 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 air, and the labeled isoprenols were suspended in SS by sonication in a Branson bath sonicator for 10 min. After sonication an aliquot was taken to verify that all of the ^{3}H-labeled precursor had been quantitatively dispersed at a concentration of 1–7 μCi/μl of SS. C6 cells were grown to near confluence in Falcon 3001 tissue culture dishes in complete medium, and after removal of the growth medium by gentle aspiration, 500 μl of labeling medium consisting of Ham's F10 and SS (final concentration, 3–5%) containing [^{3}H]GG-OH, [^{3}H]F-OH, or [^{3}H]-mevalonolactone was added. The cells were incubated with the isotopic precursors at 37°C under 5% CO_2 for the indicated interval, and the rate of formation of the labeled products was assayed as described below.

For metabolic labeling with [^{3}H]thymidine, C6 cells were grown to near confluence in Falcon 3001 tissue culture dishes as described above. Complete medium was removed and replaced with fresh medium containing lovastatin (5 μg/ml). The cells were preincubated until cell division ceased (~24 h). The medium was then removed by gentle aspiration and replaced with fresh medium containing lovastatin (5 μg/ml) or complete medium containing lovastatin (5 μg/ml) and mevalonolactone (200 μ*M*), GG-OH (10 μ*M*), or F-OH (10 μ*M*). The cells were then incubated for an additional 24 h, and [^{3}H]thymidine (final concentration, 4 μCi/ml) was added. After 4 h the cells were washed with and suspended in PBS. The cells were sedimented by centrifugation, and the PBS was removed by aspiration. The cell pellet was resuspended in ice-cold 10% trichloroacetic acid, and the denatured cell residue was sedimented by centrifugation. The cell pellet was resuspended a second time in cold trichloroacetic acid and resedimented by centrifugation. After the trichloroacetic acid was aspirated, the cell pellet was washed with diethyl ether to remove residual trichloroacetic acid. The ether was removed by aspiration after centrifugation, and the residual organic solvent was evaporated under a stream of air. The protein pellet was dissolved in 0.2 *M* NaOH, and aliquots were taken for protein content determination and for liquid scintillation counting. The aliquot for liquid scintillation counting was neutralized with 5% acetic acid before addition of the liquid scintillation cocktail.

Isolation and analysis of metabolically labeled proteins

After the C6 cells were metabolically labeled with the various isotopic precursors for 18 h, the cells were washed with ice-cold PBS, incubated for 5 min in PBS containing 2 m*M* EDTA, scraped from the dishes, and transferred to a conical tube. The cell suspension was centrifuged (200 *g*, 5 min), and the PBS was removed by aspiration. Methanol (2 ml) was added, and the suspension was sonicated to disrupt the cell pellet. The mixture was centrifuged to sediment the delipidated protein, and the pellet was reextracted twice with 2 ml of chloroform/methanol (2:1 vol/vol). After the residual organic solvent was removed from the pellet by evaporation under a stream of nitrogen, the partially delipidated protein residues were dissolved in 2% sodium dodecyl sulfate and 5 m*M* 2-mercaptoethanol. An aliquot was used to determine the amount of labeled precursor incorporated into protein, and the radiolabeled polypeptides (10–30 μg of protein) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 10.5% polyacrylamide resolving gel (Crick et al., 1994).

Pronase E digestion of metabolically labeled proteins and chromatographic identification of ^{3}H-isoprenylated cysteine residues

To identify the metabolically labeled isoprenylated cysteine residues, the protein pellets were delipidated as described above and digested extensively with Pronase E (10 mg/ml, 18 h, 37°C). The conditions for the proteolytic treatment of the metabolically labeled protein fraction and the analysis of the butanol-soluble products by reverse-phase TLC have been described in detail previously (Crick et al., 1994, 1995*a*). The chemical synthesis of farnesyl-cysteine

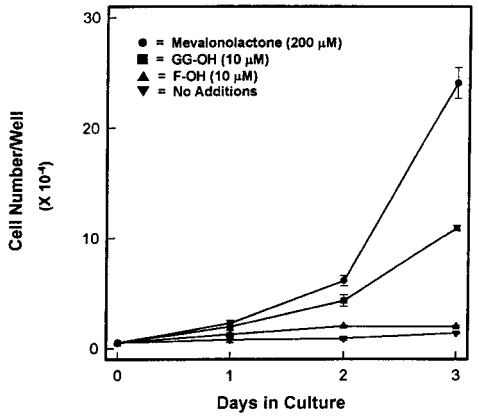

FIG. 2. Time course for growth of lovastatin-treated C6 glioma cells in the presence of mevalonate, GG-OH, and F-OH. C6 cells (5×10^3 cells per well) were plated in a 24-well tissue culture dish and allowed to attach overnight. The medium was removed by aspiration and replaced with 1 ml of complete medium containing 5 μg/ml lovastatin and 200 μM mevalonolactone, 10 μM GG-OH, or 10 μM F-OH. After the indicated interval in culture the cells were released from the dish with trypsin-EDTA and counted using a hemocytometer. All results are average ± SEM (bars) values calculated from triplicate analyses and are representative of several experiments.

(F-Cys) and geranylgeranyl-cysteine (GG-Cys) was as described by Kamiya et al. (1979) except that isopropanol was substituted for methanol when synthesizing GG-Cys. Unlabeled F-Cys and GG-Cys were detected by spraying with an anisaldehyde reagent (Dunphy et al., 1967), and radioactive compounds were located using a Bioscan System 200 Imaging Scanner.

Additional methods

Protein concentrations were assayed using a Pierce (Rockford, IL, U.S.A.) BCA Protein Assay Kit. Radioactivity was determined by liquid scintillation spectrometry in a Packard Tri-Carb 2100TR using Econo-Safe liquid scintillation cocktail (Research Products International Corp., Mt. Prospect, IL, U.S.A.). All data are average values of replicate determinations from at least three representative experiments.

RESULTS

Cell growth is restored in lovastatin-treated cells by mevalonate and GG-OH but not F-OH

Incubation of C6 glioma cells with complete medium in the presence of increasing concentrations of the HMG-CoA reductase inhibitor lovastatin resulted in a marked, concentration-dependent reduction in cell growth. Cell growth was inhibited 95–100% at lovastatin concentrations of 1.5–2 μg/ml. From these data an IC_{50} value of 0.2 μg/ml was calculated for C6 glial cells (Crick et al., 1996).

The ability of GG-OH and F-OH to restore protein geranylgeranylation or farnesylation selectively was then used as an experimental approach to examine the possibility that lovastatin blocked cell division because it depleted the F-P-P/GG-P-P pools and consequently prevented protein isoprenylation. The experimental strategy is illustrated in Fig. 1. As outlined in this metabolic scheme, the biosynthesis of F-P-P and GG-P-P can be restored specifically by the utilization of the appropriate free allylic isoprenols. This strategy could also answer the basic question addressed in this study even if F-OH and GG-OH were not converted to F-P-P and GG-P-P, but rather to other novel functional isoprenyl intermediates, which are evidently capable of serving as farnesyl and geranylgeranyl donors (Crick et al., 1994, 1995*a*).

The results presented in Fig. 2 show that when either 200 μM mevalonate or 10 μM GG-OH was supplemented in the culture medium, the G_1 arrest imposed by lovastatin (5 μg/ml) was overcome, and the cells continued to divide for at least 3 days. The increase in cell number was higher for mevalonate- compared with GG-OH-supplemented cultures, but in both cases the cells grew exponentially for 72 h. Even though F-OH can be utilized for protein farnesylation in C6 cells (Crick et al., 1995*a*), only an extremely small growth stimulus was seen when F-OH was added to the culture medium. The combination of 10 μM F-OH plus 10 μM GG-OH was no more effective than GG-OH alone. Cell growth was optimal at mevalonate concentrations of 100–200 μM and at 5–10 μM GG-OH. At concentrations >10 μM, F-OH and GG-OH inhibited cell growth, possibly owing to the formation of toxic oxidation products. Ohizumi et al. (1995) have reported that 50 μM GG-OH is a potent inducer of apoptosis in human leukemia HL-60 cells.

The relatively larger increase in cell number seen when 200 μM mevalonate was added to the culture medium compared with 10 μM GG-OH could be due to mevalonate producing a larger increase in the GG-P-P pool because of the limited solubility of the free isoprenol or because mevalonate is also a precursor for other compounds not formed from GG-OH.

A GG-P-P-based drug, PAG, has been shown to interfere with protein geranylgeranylation ($p21^{rap1}$), but not farnesylation ($p21^{ras}$) in PC-3 prostate cancer cells (Macchia et al., 1996). To obtain further evidence that GG-OH stimulated cell growth because it restored protein geranylgeranylation, the effect of PAG on the cell growth in the mevalonate- and GG-OH-supplemented cultures was tested. The data shown in Table 1 indicate that PAG (38 μM), the protein geranylgeranylation inhibitor (Macchia et al., 1996), significantly inhibited the stimulation of cell growth when mevalonate or GG-OH was added to the culture medium.

The simplest conclusion from these results is that the block in cell proliferation is overcome because GG-P-P synthesis and consequently protein geranylgeranylation are restored by addition of mevalonate or GG-OH to the culture medium. Although farnesylated proteins may also be involved in the regulation of cell growth, the results in Fig. 2 show that restoring protein farnesylation alone did not provide the substantial

TABLE 1. *Restoration of growth in lovastatin-treated C6 glioma cells by mevalonate and GG-OH is blocked by the GG-P-P-based inhibitor PAG*

Supplement to growth medium	C6 cells/dish ($\times 10^{-3}$)	
	No addition	PAG (38 μM)
None	20.5	22.0
Mevalonate (200 μM)	78.3	19.3
GG-OH (10 μM)	82.3	33.0

The details of the procedures for cell culture and counting cell numbers are described in Materials and Methods.

growth stimulus seen when protein geranylgeranylation was restored by GG-OH or mevalonate supplementation. This result is in agreement with the previous observation that if F-P-P is formed from F-OH, only an extremely small fraction of this pool of F-P-P is converted to GG-P-P (Crick et al., 1995*a*).

GG-OH and mevalonate stimulate [^{3}H]thymidine incorporation in lovastatin-treated cells

To obtain a second line of evidence that GG-OH promoted entry of lovastatin-treated C6 cells into the cell cycle, the rate of [^{3}H]thymidine incorporation, as an estimation of DNA synthesis, was compared after addition of mevalonate or GG-OH to the culture medium. As seen in Table 2, the rate of [^{3}H]thymidine incorporation was substantially higher in lovastatin-treated cells when 200 μM mevalonate was supplemented to the culture medium compared with control cells. Consistent with GG-OH promoting entry into the S phase of the cell cycle, the rate of DNA synthesis was similar in cells supplemented with mevalonate or 10 μM GG-OH. Addition of F-OH to the culture medium produced a stimulation in [^{3}H]thymidine labeling slightly higher than no additions but significantly lower than mevalonate or GG-OH. Restoring protein farnesylation may provide part of the stimulatory response for DNA replication, but based on the results in Fig. 2 this effect is not sufficient for cell proliferation. The relatively low [^{3}H]thymidine incorporation observed in control cells is probably due to the process of DNA repair because there was no increase in cell number under those conditions.

TABLE 2. *Stimulation of [^{3}H]thymidine incorporation by mevalonate and GG-OH in lovastatin-treated C6 cells*

Addition to growth medium	[^{3}H]Thymidine incorporation (% of control)
None	48
Mevalonate (200 μM)	100
GG-OH (10 μM)	87
F-OH (10 μM)	67

The procedures for cell culture, metabolic labeling, and assay of [^{3}H]thymidine labeling are described in Materials and Methods. The value for 100% of the control was 547,586 ± 10,038 dpm/mg of protein (mean ± SEM).

Thus, the increases in cell number and stimulation of DNA synthesis seen when lovastatin-treated C6 cells are incubated in a medium supplemented with GG-OH indicate that lovastatin blocks cell proliferation because of the depletion of GG-P-P pools required for the geranylgeranylation of one or more proteins essential for promoting entry of C6 cells into the S phase of the cell cycle.

Characterization of C6 proteins metabolically labeled by [^{3}H]mevalonate and [^{3}H]GG-OH in lovastatin-treated C6 glioma cells

To verify that exogenous mevalonate and GG-OH could restore protein geranylgeranylation in lovastatin-treated cells, C6 cells were incubated with [^{3}H]-mevalonate or [^{3}H]GG-OH in the presence of lovastatin, and the metabolically labeled proteins and the isoprenylated amino acid residues were analyzed. The gel patterns in Fig. 3 show that a set of polypeptides in the size range of small-molecular-weight GTP-binding proteins (19–27 kDa) were metabolically labeled by both isotopic precursors. Although the metabolically labeled proteins are in the same size range of well-characterized G proteins, specific geranylgeranylated proteins were not identified in this study.

As observed in earlier studies (Crick et al., 1994, 1995*a*), another protein with an apparent molecular mass very similar to the size of the smaller isoform of CNPase (46 kDa) was also metabolically labeled by

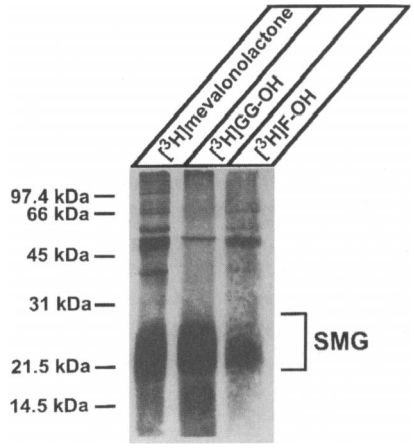

FIG. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of proteins metabolically labeled by [^{3}H]-mevalonate, [^{3}H]F-OH, and [^{3}H]GG-OH in lovastatin-treated C6 glioma cells. C6 cells were metabolically labeled with the indicated isotopic precursor for 18 h as described in Materials and Methods. The delipidated protein fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 10% polyacrylamide resolving gel. The calibration markers are indicated on the left, and the designation SMG refers to the range of the small-molecular-mass (19–27-kDa) GTP-binding proteins. The results are representative of at least three separate experiments.

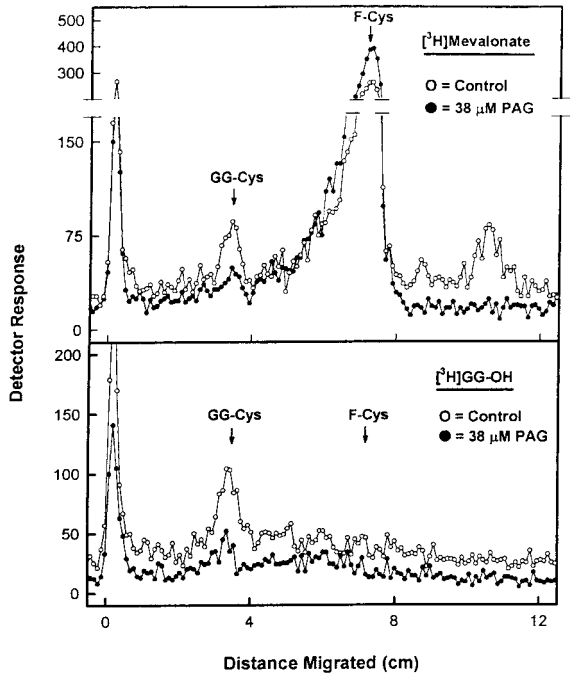

FIG. 4. Analysis of isoprenylated cysteine residues in C6 glioma proteins metabolically labeled by [^{3}H]mevalonate and [^{3}H]GG-OH in the presence and absence of PAG. Near-confluent cultures of C6 cells were incubated in Falcon 3001 tissue culture dishes with the indicated isotopic precursor in the presence or absence of 38 μM PAG for 18 h. The labeled protein fractions were isolated and digested with Pronase E as described in Materials and Methods. The labeled products were extracted with 1-butanol (saturated with water) and analyzed on C18 reverse-phase TLC plates developed in acetonitrile/water/acetic acid (75:25:1 by volume). Radioactive zones were detected with a Bioscan Imaging Scanner System 200-IBM. The arrows indicate the position of authentic F-Cys and GG-Cys. The results are representative of at least three separate experiments.

[^{3}H]GG-OH and [^{3}H]mevalonate. Although it is not certain that the 46-kDa protein labeled via [^{3}H]GG-OH in this study is CNPase, the myelin protein has been shown to be metabolically labeled by incubating C6 cells (Braun et al., 1991) and rat brainstem slices (Sepp-Lorenzino et al., 1994) with [^{3}H]mevalonate.

The presence of cysteinyl-linked isoprenyl groups in the proteins metabolically labeled when C6 cells were incubated with [^{3}H]GG-OH and [^{3}H]mevalonate was confirmed by showing that [^{3}H]GG-Cys was released from the delipidated protein fractions by digestion with Pronase E (Fig. 4). The metabolically labeled product(s) remaining at the origin in this chromatographic system could be partially degraded mono- or digeranylgeranylated Cys-Cys or Cys-X-Cys sequences as found in Rab proteins (Seabra et al., 1992; Farnsworth et al., 1994). Consistent with this possibility, when Rab1a, a protein that has a Cys-Cys carboxy terminus, was [^{3}H]geranylgeranylated in vitro by incubation with [^{3}H]GG-P-P and then digested with Pronase E, a radiolabeled product that remained at the origin of the reverse-phase TLC plates developed as described in Materials and Methods was seen (data not shown).

The inclusion of PAG (38 μM) in the culture medium inhibited the incorporation of [^{3}H]GG-OH and [^{3}H]mevalonate into GG-Cys in C6 cells as assessed by reverse-phase TLC analysis of products released from cellular proteins by exhaustive Pronase E digestion (Fig. 4).

It is possible that one or more of the proteins labeled by [^{3}H]GG-OH and [^{3}H]mevalonate are responsible for the growth-stimulus-promoting entry into the S phase of the cell cycle in lovastatin-treated C6 cells. The polypeptides in the small-molecular-weight GTP-binding protein range, the major proteins labeled via [^{3}H]GG-OH, may be the key regulatory proteins involved in the control of cell cycle progression in C6 cells.

DISCUSSION

In this article we have used a novel experimental approach in an attempt to identify the mevalonate-derived product required for the proliferation of glial cells (Volpe and Obert, 1983; Bhat and Volpe, 1984; Volpe et al., 1985; Langan and Volpe, 1986, 1987). Identification of this product should provide new insight into the regulation of cell cycle progression in glial cells.

These studies have taken advantage of an experimental strategy, based on the observation that C6 glioma cells utilize GG-OH and F-OH for protein isoprenylation (Crick et al., 1994, 1995*a*), to determine if cell growth is arrested by lovastatin because of a depletion of the F-P-P and GG-P-P pools. As illustrated in Fig. 1, when mevalonate biosynthesis is blocked by lovastatin, free GG-OH and F-OH can restore the pools of GG-P-P and F-P-P required for the posttranslational modification of farnesylated and geranylgeranylated proteins. Thus, this "salvage" pathway provides a means of restoring protein farnesylation or geranylgeranylation selectively by adding exogenous F-OH or GG-OH to the culture medium.

The results of these cellular studies strongly suggest that one or more geranylgeranylated proteins are essential for entry of C6 cells into the cell cycle. Although a 46-kDa protein, similar in size to CNPase, is geranylgeranylated under these conditions, it is more likely that the critical isoprenylated regulatory proteins are in the set of polypeptides in the size range of the small-molecular-weight GTP-binding proteins (19–27 kDa), metabolically labeled during incubations with [^{3}H]GG-OH. The geranylgeranylated G proteins Rho, Rac, and CDC42 have been implicated in the regulation of cell cycle progression in other experimental cell systems (Olson et al., 1995; Symons, 1996), possibly by affecting the degradation of $p27^{Kip1}$ (Hirai et al., 1997).

With this study on lovastatin-treated C6 cells, there

is now a growing body of evidence documenting the physiological significance of the salvage pathway for GG-OH and F-OH in mammalian cells. Exogenously supplied GG-OH restored growth in simvastatin-treated arterial (Corsini et al., 1993) and bronchial (Vigano et al., 1995) myocytes. More recently, addition of GG-OH to the culture medium has been shown to restore platelet-derived growth factor receptor tyrosine phosphorylation (McGuire et al., 1996) and cell cycle progression (Vogt et al., 1996) when mevalonate synthesis was blocked with lovastatin in NIH-3T3 cells. The same laboratory has shown that GG-OH potentiates the inhibition of H-Ras processing caused by lovastatin in NIH-3T3 cells transfected with oncogenic H-Ras-CVSL (McGuire and Sebti, 1997). The ability of GG-OH to promote entry of UT-2 cells (Crick et al., 1997*a*), which are unable to synthesize mevalonate owing to a genetic defect in HMG-CoA reductase (Mosley et al., 1983), into the S phase of the cell cycle provides evidence that this pathway is an alternate mechanism for synthesizing physiologically significant levels of GG-P-P, or another "activated" geranylgeranyl donor that is recognized by protein isoprenyltransferases. In addition to the cellular effects of GG-OH, an important physiological role for F-OH is also supported by the evidence that the free isoprenol stimulates the regulated proteolysis of HMG-CoA reductase (Bradfute and Simoni, 1994; Correll et al., 1994; Miegs et al., 1996; Miegs and Simoni, 1997).

The role of F-OH in the regulation of HMG-CoA reductase activity and the evidence that F-OH and GG-OH can serve as precursors for "activated" isoprenyl donors in protein isoprenylation emphasize the importance of elucidating the enzymatic mechanism by which the allylic isoprenols are converted to F-P-P and GG-P-P or previously unidentified isoprenyl intermediates. This pathway may be a salvage mechanism for the reutilization of F-OH and GG-OH produced by turnover of GG-P-P and F-P-P by membrane-bound pyrophosphatases (Bansal and Vaidya, 1994) and possibly the degradation of isoprenylated proteins. An enzyme activity capable of cleaving thioether bonds in F-Cys, reported for pig liver microsomes (Park et al., 1994), could be involved in this catabolic process. If a similar mechanism exists for *trans,trans,cis*-GG-OH produced by the turnover of *trans,trans,cis*-GG-P-P, this stereoisomer would be a unique precursor for the biosynthesis of dolichyl phosphate (Waechter, 1989; Grunler et al., 1994).

Squalestatin 1 (Fig. 1) is a selective inhibitor of squalene synthase (Baxter et al., 1992; Bergstrom et al., 1993; Hasumi et al., 1993; Thelin et al., 1994; Crick et al., 1995*b*), the enzyme catalyzing the NADPH-dependent biosynthesis of squalene from two molecules of F-P-P (Agnew, 1985; Grunler et al., 1994). The observation that squalestatin 1 blocks the incorporation of labeled F-OH into squalene and sterol in C6 glioma and African green monkey kidney (CV-1) cells strongly suggests that F-OH and GG-OH are converted to the pyrophosphate intermediates (Crick et al., 1995*a*). The inhibitory effect of NB-598, a squalene epoxidase inhibitor, on the incorporation of [^{3}H]F-OH into cholesterol in rat retinas also indicates that the isotopic precursor was incorporated via the conventional pathway (Fliesler and Keller, 1995).

A preliminary report has indicated that rat liver microsomes catalyze the conversion of F-OH to F-P-P in the presence of CTP at a very modest rate (Westfall et al., 1997). However, the nucleotide and isoprenol specificities of this enzyme system have not been thoroughly characterized. CTP-dependent F-OH kinase activity had previously been detected in a cell extract from *B. braunii* (Inoue et al., 1994). It will be important to determine if these phosphorylation reactions are a nonspecific activity of CTP-mediated dolichol kinase (Allen et al., 1978; Burton et al., 1979; Sumbilla and Waechter, 1985).

As yet there has been no documentation of the mono- or pyrophosphorylation of GG-OH in mammalian cells, although the archebacterium *Sulfolobus acidocaldarius* has been reported to contain GG-OH kinase activity (Ohnuma et al., 1996). Recently, it has been proposed that GG-OH is converted to a novel geranylgeranyl donor, referred to as GG-X, because these investigators were unable to detect metabolically labeled GG-P-P when H-Ras-CVLS-transformed NIH-3T3 cells were incubated with [^{3}H]GG-OH (McGuire and Sebti, 1997).

In summary, several lines of evidence are presented supporting the conclusion that GG-P-P is a critical mevalonate-derived intermediate that is essential for glial cell proliferation. Based on this study and others cited above, GG-P-P is required for the posttranslational modification of geranylgeranylated proteins involved in the regulation of the cell cycle. Important future goals will be to establish the mechanistic details of how GG-OH is converted to GG-P-P or a novel isoprenyl donor and the precise function of the geranylgeranylated protein(s) in controlling glial cell cycle progression. It should also be possible to apply the same experimental strategy described here to investigate if HMG-CoA reductase inhibitors block glial cell differentiation (Volpe and Obert, 1983) as a result of depleting F-P-P and/or GG-P-P pools, consequently preventing isoprenylation of a different group of regulatory proteins required for the induction of the differentiation process.

Acknowledgment: This work was supported by grant GM36065 from the National Institutes of Health awarded to C. J. W.

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