Biochemical Pharmacology, Vol. 54, pp. 113–120, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00151-2

Inhibition of Type I and Type II Geranylgeranyl-Protein Transferases by the Monoterpene Perillyl Alcohol in NIH3T3 Cells

Zhibin Ren, Charles E. Elson† and Michael N. Gould*‡*

DEPARTMENTS OF *HUMAN ONCOLOGY AND †NUTRITIONAL SCIENCES, UNIVERSITY OF WISCONSIN-MADISON, MADISON, WI 53792, U.S.A.

ABSTRACT. The monoterpene perillyl alcohol has anticancer activities that include both prevention and treatment of a wide variety of cancers in animal models. In purified enzyme studies, perillyl alcohol inhibited farnesyl-protein transferase and type I geranylgeranyl-protein transferase. However, whether and which of the polyprenyl-protein transferases is inhibited by perillyl alcohol *in vivo* is not known. The previously reported monoterpene-induced inhibition of the incorporation of [^{14}C]mevalonolactone into proteins in cultured cells could be due to an inhibition of one or several enzymes in the mevalonate pathway or to changes in the levels of protein substrates for isoprenylation. In the current study, we first analyzed the levels of individual phosphorylated isoprenoid intermediates between mevalonate and geranylgeranyl pyrophosphate in NIH3T3 cells labeled for 4 hr with [^{14}C]mevalonolactone and found that perillyl alcohol did not inhibit the synthesis of these intermediates. Next, proteins including Ras, RhoA, and Rab6 were immunoprecipitated from NIH3T3 cells. Perillyl alcohol was found to inhibit the incorporation of [^{14}C]mevalonolactone into RhoA and Rab6 but not Ras protein. The cellular levels of these three proteins were constant over the 4-hr treatment period. Finally, the distribution of Ras, Rap1, and Rab6 proteins between the aqueous and the detergent-enriched phases was measured. Rap1 and Rab6 but not Ras from perillyl alcohol-treated NIH3T3 cells accumulated in the aqueous phase. Thus, we conclude that perillyl alcohol can inhibit the *in vivo* prenylation of specific proteins by type I and type II geranylgeranyl-protein transferases but not farnesyl-protein transferase in NIH3T3 cells. BIOCHEM PHARMACOL **54**;1:113–120, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. farnesyl-protein transferase; geranylgeranyl-protein transferases; monoterpenes; perillyl alcohol; cancer chemotherapy; cancer chemoprevention

The monoterpene POH§ is effective in both preventing and treating a variety of rodent cancers [1–3]. Dietary POH induced the regression of 75% of advanced, chemical carcinogen-initiated rat mammary carcinomas [2], and the majority of these regressions were complete. Based on this and other rodent [1–3] and cell line [4] data, POH is currently undergoing phase I clinical trials in advanced cancer patients [5]. The cellular activities of POH include the inhibition of protein isoprenylation [4, 6] and the syntheses of CoQ and cholesterol [7]. In addition, in dietary monoterpene-treated regressing tumors the induction of several genes, including mannose 6-phosphate/insulin-like growth factor II receptor, has been observed [1, 8]. In the current study, we focus on the inhibition of protein isoprenylation by monoterpenes in order to better define this activity in a cellular setting.

Protein isoprenylation is one form of post-translational modification of cellular proteins. The isoprenylation signal of a protein is a short amino acid sequence located at its carboxyl end. There are at least three enzymes that catalyze the protein isoprenylation reaction (reviewed in Ref. 9). If a protein ends with CAAX at its carboxyl-terminus, where C is a cysteine, A is an aliphatic amino acid, and X is a serine, methionine, alanine, or glutamine, the protein will be modified with a 15-carbon isoprene compound, FPP, catalyzed by the enzyme called FPTase. Ras, nuclear lamins, transducin-γ, and rhodopsin kinase are examples of this family of proteins. If the X in the carboxyl-terminal CAAX box of a protein is a leucine or isoleucine, the protein will be modified with a 20-carbon isoprene compound, GGPP, catalyzed by the enzyme type I GGPTase. Examples of this family of proteins include Rac, RhoA, Rap1, and G protein γ-subunit. If the carboxyl-terminus of a protein is CC, CXC, or CCXX, where C is a cysteine and X is any amino acid, the protein will be modified with GGPP catalyzed by

‡ Corresponding author: Dr. Michael N. Gould, University of Wisconsin-Madison, Department of Human Oncology, K4/332, 600 Highland Ave., Madison, WI 53792. Tel. (608) 263-6615; FAX (608) 263-9947; E-mail: mgould@macc.wisc.edu

§ *Abbreviations*: BCA, bicinchoninic acid; CoQ, coenzyme Q; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; FPTase, farnesyl-protein transferase; GGPP, geranylgeranyl pyrophosphate; GGPTase, geranylgeranyl-protein transferase; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; MVA-P, mevalonate phosphate; and MVA-PP, mevalonate pyrophosphate.

Received 9 September 1996; accepted 28 January 1997.

the enzyme type II GGPTase. Rab protein family members are modified by this enzyme.

The isoprenylation of several oncoproteins including Ras suggests new targets for cancer therapeutics [10]. For example, isoprenylation of Ras is required for its transforming activity [11, 12]; thus, FPTase inhibitors that block this modification of Ras protein have the potential to be effective in cancer therapy [13–15]. Recently, other isoprenylated small G proteins, such as Rac1, RhoA, and RhoB, have also been shown to have transforming properties [16–18]. Rac1 and RhoA are geranylgeranylated by type I GGPTase [9], suggesting the possibility that type I GGPTase may also be a target for anticancer drug development.

In purified enzyme systems, POH inhibited FPTase and type I GGPTase [6]. The effect of POH on type II GGPTase has yet to be investigated. The ability of monoterpenes to specifically inhibit polyprenyl-protein transferases *in vivo* remains to be demonstrated. Crowell *et al.* [19] showed that monoterpenes reduce the amount of isoprenylated small G proteins in cultured cells labeled with [^{14}C]mevalonolactone. However, it is not clear whether monoterpenes act on the initial common branch of the mevalonate pathway or specifically on one or more of the polyprenyl-protein transferases. Monoterpenes have also been shown to down-regulate Ras [20]. Yet, whether monoterpenes can down-regulate other small G proteins and whether this possible down-regulation correlates with the ability of monoterpenes to reduce the amount of isoprenylated small G proteins are unknown.

MATERIALS AND METHODS

Materials

POH was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Dulbecco's modified Eagle's medium and calf serum were from GIBCO (Grand Island, NY) and HyClone Laboratories (Logan, UT), respectively. Lovastatin, provided by Alfred Alberts of Merk, Sharpe & Dohme, was converted to the acid before use [21]. (*R*, *S*)-[2-^{14}C]Mevalonolactone was purchased from DuPont-New England Nuclear (Boston, MA). *Trans*, *trans*-[1-^{3}H]FPP and all-*trans*-[1-^{3}H]GGPP were from American Radiolabeled Chemicals (St. Louis, MO). The BCA protein assay kit was from Pierce (Rockford, IL). Tris and other electrophoresis reagents were from Bio-Rad (Hercules, CA). Wild type H-Ras, CVLL-H-Ras, and Rab3a proteins were gifts from Panvera (Madison, WI). Rat anti-H-Ras (clone Y13-259) IgG_1, rabbit anti-RhoA IgG, rabbit anti-Rab6 IgG, protein A-Agarose, and protein A/G PLUS-Agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-pan-Ras IgG_{2a} was from Oncogene Science (Cambridge, MA) and Immobilon™ PVDF membrane was from Millipore (Bedford, MA). Anti-mouse and anti-rabbit IgG/horseradish peroxidase were from Amersham (Arlington Heights, IL). Creatine phosphate, creatine phosphokinase, and RNase were from Boehringer Mannheim (Indianapolis, IN) and the ATP used in the cell lysate assay was from Pharmacia Biotech (Piscataway, NJ). Acetonitrile and petroleum ether (30–60°) were from J. T. Baker (Glen Ellyn, IL) and Fisher Scientific (Itasca, IL), respectively. All other biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Cell Culture

NIH3T3 cells from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and maintained in 100-mm dishes at 37° in a humidified 5% CO_2 atmosphere.

Labeling Cells with [^{14}C]Mevalonolactone

NIH3T3 cells (80–90% confluency) were treated with 30 μM lovastatin (an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase) for 18–24 hr to deplete the endogenous pool of mevalonate and then incubated in fresh medium containing 30 μM lovastatin and 215 μM (*R*, *S*)-[2-^{14}C]mevalonolactone (58 mCi/mmol) for 3 or 4 hr in the absence or presence of POH. The medium was then decanted, the cells were scraped into ice-cold PBS and washed twice, and the cell pellets were stored at −20° for further analysis.

Measurement of Polyprenyl Pyrophosphate Levels by HPLC

The phosphorylated isoprenoid intermediates from [^{14}C]mevalonolactone-labeled NIH3T3 cells were extracted and analyzed as described by Watson *et al.* [22] with slight modifications. NIH3T3 cells collected from five confluent plates were lysed with 0.45 mL of hot (75°) 0.5 M NH_4OH, 75% ethanol solution. The denatured cell suspension was then extracted (three times) with 0.45 mL of petroleum ether (30–60°). Insoluble protein in the ethanolic phase was then pelleted by centrifugation at 10,000 *g* for 15 min. The supernatant was saved, and the pellet was re-extracted with 0.1 mL of hot 0.5 M NH_4OH, 75% ethanol solution. The supernatants were pooled, and the volume was reduced to 0.15 mL by a stream of nitrogen gas. All 0.15 mL of supernatant was applied to HPLC analysis using a Hamilton PRP-1 (0.4 × 15 cm) column. A 30-min linear gradient of 15 to 60% acetonitrile in 0.01 M tetrabutylammonium phosphate, pH 7.5, was run at a flow rate of 2 mL/min. The effluent was collected as 1-mL fractions, and the radioactivity of each fraction was determined by scintillation counting.

Immunoprecipitation of Ras, RhoA, and Rab6 Proteins

[^{14}C]Mevalonolactone-labeled NIH3T3 cells (1×10^7) were lysed with 500 μL of ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mM sodium or-

thovanadate and 0.15 to 0.3 trypsin inhibitor unit/mL aprotinin), and the cellular debris was pelleted by centrifugation at 10,000 *g* for 15 min. The supernatant was collected, and the protein concentration was determined by BCA assay. The supernatant was diluted to 1–3 mg protein/mL with RIPA buffer, and then 1 mL was taken for immunoprecipitation. Antibody (1.5 μg of rat anti-H-Ras IgG_1 or rabbit anti-RhoA IgG or rabbit anti-Rab6 IgG) was added to and incubated with the 1 mL extract at 4° for 3 hr, after which 35 μL agarose conjugate (Protein A-Agarose or Protein A/G PLUS-Agarose) was added, and incubation was continued overnight. The resulting immunoprecipitates were centrifuged and washed four times with RIPA buffer. Samples were then boiled for 4 min in a buffer containing 62 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol and analyzed by SDS–PAGE. The radioactive bands on the SDS–PAGE gels were quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

NIH3T3 Cell Lysate Assay

Assays of FPTase and type I and type II GGPTases were performed as described by Lutz *et al.* [23] except that NIH3T3 cell lysate was used instead of rabbit reticulocyte lysate. Confluent NIH3T3 cells were scraped into and washed three times with ice-cold PBS. The cells were then pelleted and lysed with ice-cold double-distilled water. After centrifugation at 10,000 *g* for 15 min, the supernatant was collected and the protein concentration was determined by BCA assay. In the presence or absence of protein prenylation inhibitors, the supernatant containing 10–20 μg protein was incubated at 37° for 1 hr with 0.4 μM [^{3}H]FPP (25 Ci/mmol) or 0.4 μM [^{3}H]GGPP (25 Ci/mmol), 2 μg of H-Ras or CVLL-H-Ras or Rab3a protein, 1 mM $MgCl_2$, 1 mM dithiothreitol, 3 mM ATP, 10 mM creatine phosphate, 50 μg/mL creatine phosphokinase, and 0.4 mg/mL RNase in a final volume of 50 μL. Then 30 μL from each reaction was taken and analyzed by SDS–PAGE and fluorography.

Detection of Ras, RhoA, and Rab6 Proteins by Western Blot

Confluent NIH3T3 cells treated with POH for 4 hr were harvested and washed three times with ice-cold PBS. The pelleted cells were then lysed with ice-cold RIPA buffer, and protein concentrations were determined by BCA assay. One hundred micrograms of protein from each sample was separated on a 15% SDS–PAGE gel, and then the proteins were transferred to Immobilon™ PVDF membrane. The membrane was blocked with 5% non-fat dry milk in TBST (0.1 M Tris, pH 7.5, 0.9% NaCl, 0.1% Tween-20, by vol.) for 30 min. After washing with TBST, the membrane was incubated for 1 hr with the first antibody (mouse anti-pan-Ras IgG_{2a} or rabbit anti-RhoA IgG or rabbit anti-Rab6 IgG, 1/3000 dilution in 0.5% milk TBST). The membrane was washed again with TBST and then incubated for 50 min with the secondary antibody (anti-mouse or anti-rabbit IgG/horseradish peroxidase, 1/2000 dilution in 0.5% milk TBST). After the final wash with TBST, the proteins on the membrane were detected with the Amersham ECL™ system.

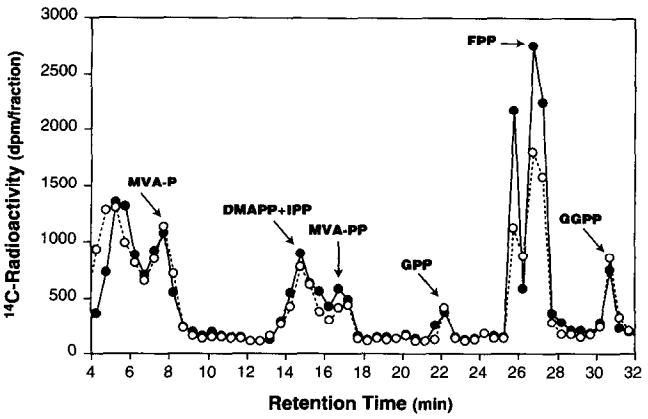

FIG. 1. Effect of POH on common intermediates of the mevalonate metabolism pathway. Phosphorylated isoprenoid intermediates from [^{14}C]mevalonolactone-labeled control (○) or 1 mM POH-treated (●) NIH3T3 cells were extracted and analyzed by HPLC as described in Materials and Methods.

Separating Proteins into Aqueous and Detergent-Enriched Phases

The isoprenylated and unprocessed forms of Ras, Rap1, and Rab6 proteins were separated into the detergent-enriched phase and the aqueous phase of 1% Triton X-114 TBS (20 mM Tris and 150 mM NaCl, pH 7.5) buffer as described by Gutierrez *et al.* [24]. The 1 mM POH (43 hr)- or 30 μM lovastatin (24 hr)-treated and control NIH3T3 cells were harvested and washed as described above. The cell pellet was lysed with ice-cold 1% Triton X-114 TBS buffer. The cell debris was pelleted by centrifugation and the supernatant was incubated at 37° for 5 min. The turbid solution was centrifuged at 16,000 *g* for 2 min, and the upper (aqueous) and the lower (detergent-enriched) phases were separated. Ras, Rap1, and Rab6 levels in the aqueous phase and the detergent-enriched phase were analyzed by SDS–PAGE and western blot as described above.

RESULTS

Effect of POH on the Levels of Polyprenyl Pyrophosphates

To investigate the potential effects of POH on enzymes between mevalonate and GGPP in the mevalonate metabolism pathway, NIH3T3 cells were labeled with [^{14}C]mevalonolactone, and the polyprenyl pyrophosphate intermediates were extracted and analyzed by HPLC. As shown in Fig. 1, the HPLC system separated all polyprenyl pyrophosphate intermediates from mevalonate to GGPP with the exception of the isomers DMAPP and IPP, which had the

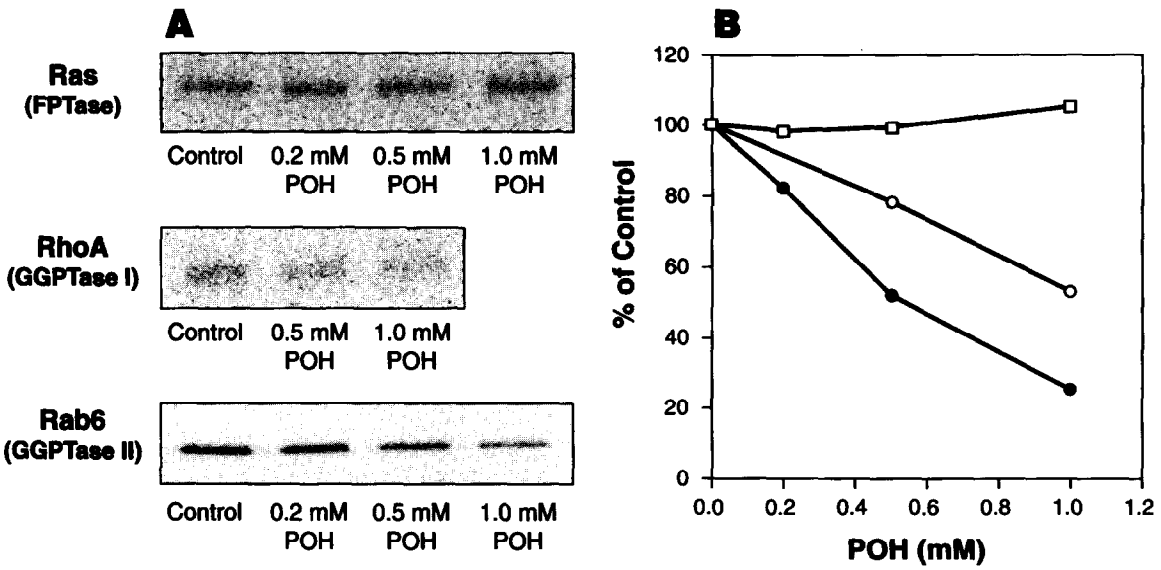

FIG. 2. Effects of POH on FPTase and type I and type II GGPTases in cultured cells. Ras, RhoA, and Rab6 proteins were immunoprecipitated from [^{14}C]mevalonolactone-labeled control or 0.2 to 1.0 mM POH-treated NIH3T3 cells. The proteins were analyzed by SDS–PAGE. The dried gels containing Ras, RhoA, and Rab6 proteins were exposed to phosphor screens for 48, 72, and 24 hr, respectively. Panel A shows the PhosphorImager scans of the exposed phosphor screens. Panel B presents the quantitations of radioactive Ras (□), RhoA (○), and Rab6 (●) protein bands from the same experiment as shown in Panel A done with the ImageQuant computer program. The 1 mM concentration data are similar to those obtained in three other independent experiments.

same retention time. When the levels of individual polyprenyl pyrophosphate intermediates in 1 mM POH-treated cells were compared with those in control cells, no difference was found with the exception of a 50% elevation of the FPP peak and the unidentified peak immediately preceding it.

Effect of POH on Polyprenyl-Protein Transferases in NIH3T3 Cells

To investigate which of the three polyprenyl-protein transferases are inhibited by POH, we assayed the prenylation of Ras, RhoA, and Rab6 proteins from [^{14}C]mevalonolactone-labeled NIH3T3 cells. Ras, RhoA, and Rab6 proteins are prenylated by FPTase, type I GGPTase, and type II GGPTase, respectively. As shown in Fig. 2 (A and B), isoprenylation of RhoA and Rab6 was inhibited by POH with IC_{50} values of 1.0 and 0.5 mM, respectively. However, isoprenylation of Ras was not inhibited by POH as high as 1 mM. The lack of activity of POH against Ras isoprenylation was not related to the experimental protocol in that B581, an FPTase inhibitor [25], was shown to inhibit Ras isoprenylation while not inhibiting RhoA isoprenylation in the same assay system (data not shown).

To explore the possibility that the 50% increase of FPP, one of the co-substrates for Ras isoprenylation, in POH-treated cells negated the POH inhibition of FPTase (Ras isoprenylation), we ran the following reconstitution experiments. First, additional [^{14}C]mevalonolactone was used to label the control cells to mimic the FPP level increase in POH-treated cells. As more [^{14}C]mevalonolactone was used to label the cells, more FPP was produced. As concentrations increased from 110 to 320 μM [^{14}C]mevalonolactone (58 mCi/mmol), the FPP levels increased linearly (Fig. 3). This study was then extended to determine whether the higher FPP level resulted in changes in

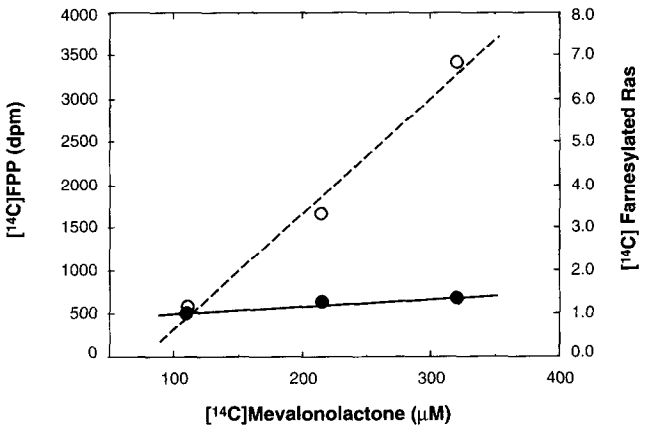

FIG. 3. FPP levels and Ras farnesylation. NIH3T3 cells were labeled with various amounts of [^{14}C]mevalonolactone (110, 215, and 320 μM). FPP was extracted and analyzed by HPLC, and Ras protein was immunoprecipitated and analyzed by SDS–PAGE as described in Materials and Methods. The figure shows the regression lines of the [^{14}C]FPP level (○) and the relative [^{14}C]Ras level (●) from [^{14}C]mevalonolactone-labeled cells versus the amount of [^{14}C]mevalonolactone used in the culture medium. The relative [^{14}C]Ras level in cells labeled in medium containing 110 μM [^{14}C]mevalonolactone is defined as 1. The correlation coefficients for the [^{14}C]FPP line and the [^{14}C]Ras line are 0.991 and 0.959, respectively. Similar results of relative amounts of radiolabeled FPP levels were obtained using [^{3}H]mevalonolactone to label the cells.

isoprenylated Ras levels. NIH3T3 cells were labeled with 110, 215, and 320 μM [^{14}C]mevalonolactone, and Ras was immunoprecipitated and analyzed on an SDS–PAGE gel. The amount of isoprenylated Ras was not altered significantly under these conditions (Fig. 3). Given that 215 μM [^{14}C]mevalonolactone was used in the experiment described in Figs. 1 and 2, we had estimated from Fig. 3 that labeling control cells with 258 μM [^{14}C]mevalonolactone would provide the same level of FPP found in 1 mM POH-treated cells labeled with 215 μM [^{14}C]mevalonolactone. However, the amount of isoprenylated Ras in 215 μM [^{14}C]mevalonolactone-labeled cells was almost the same as that in 258 μM [^{14}C]mevalonolactone-labeled cells (Fig. 3). This indicates that the 50% increase in FPP levels in POH-treated cells would not result in a higher level of isoprenylated Ras, and the lack of effect of POH on FPTase was not likely due to the observed increase in FPP.

Effects of POH on FPTase and on Type I and Type II GGPTases in NIH3T3 Cell Lysate

The result from Fig. 2 shows that POH inhibits type I and type II GGPTases but not FPTase in cultured NIH3T3 cells. To verify our *in vivo* results, we used NIH3T3 cell lysate as the source of polyprenyl-protein transferases to test the effects of POH on these enzymes. Protein substrates were added to the lysate and labeled with [^{3}H]FPP or [^{3}H]GGPP directly. Wild type H-Ras, CVLL-Ras (wild type H-Ras protein with a modification at the C-terminus so that the last residue becomes L), and Rab3a are the substrates that were used for FPTase, type I GGPTase, and type II GGPTase, respectively. As shown in Fig. 4 (A and B), FPTase, type I GGPTase, and type II GGPTase were inhibited 14, 62 and 72%, respectively, by 1 mM POH. As a control, the FPTase inhibitor B581 [25] inhibited FPTase by 91% and type I and type II GGPTase by only 30 and 17%, respectively (Fig. 4, C and D).

Effects of POH Treatment on Ras, RhoA, or Rab6 Protein Levels in NIH3T3 Cells

To investigate the possibility that POH decreases the isoprenylated RhoA and Rab6 levels by down-regulating these proteins rather than inhibiting type I and type II GGPTases, we measured RhoA, Rab6, and Ras levels in POH-treated NIH3T3 cells by western blot. As shown in Fig. 5, POH treatment for 4 hr, which was the labeling time used in previous [^{14}C]mevalonolactone labeling studies, did not change Ras, RhoA, or Rab6 protein levels.

Effects of POH Treatment on the Ratios of the Isoprenylated Form to the Unprocessed Form of Proteins in NIH3T3 Cells

Since POH inhibits type I and type II GGPTase, we hypothesized that it could change the ratio of geranylgeranylated and unprocessed forms of these proteins. We used

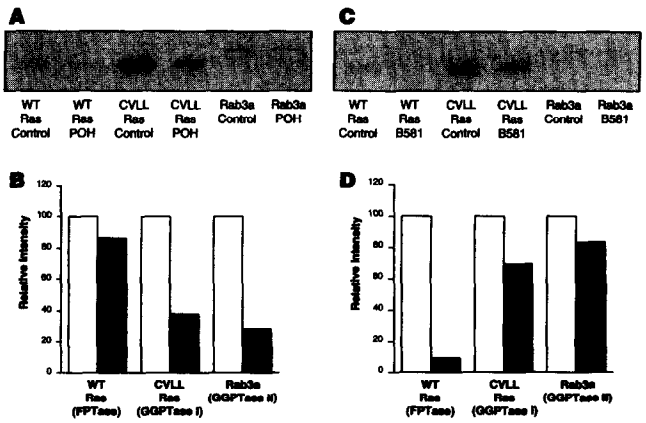

FIG. 4. Effects of POH on FPTase and type I and type II GGPTases in NIH3T3 cell lysate. Wild type (WT) Ras was labeled with [^{3}H]FPP, and CVLL-Ras and Rab3a were labeled with [^{3}H]GGPP in NIH3T3 cell lysate as described in Materials and Methods. Panels A and C show the SDS–PAGE and fluorography analysis of the labeled proteins. The quantitations of bands in the same experiment from panels A and C are shown in panels B and D, respectively. Panels A and B present results from POH treatment, while panels C and D present data from B581 (a known FPTase inhibitor) treatment. Open bars, control enzyme reactions; closed bars, enzyme reactions in the presence of 1 mM POH (panel B) or 1 μM B581 (panel D). Similar results were obtained in a second independent experiment.

Ras as a model protein that is farnesylated by FPTase, and Rap1 and Rab6 as models of the proteins that are geranylgeranylated by type I GGPTase and type II GGPTase, respectively. In our assay system, isoprenylated Ras, Rap1, and Rab6 are found in the detergent-enriched phase, while unprocessed Ras, Rap1, and Rab6 are located in the aqueous phase. POH treatment for 43 hr resulted in an accumulation of Rap1 and Rab6 proteins in the aqueous phase (Fig. 6). The same accumulations were observed when cells were treated with lovastatin. POH treatment did not result in an accumulation of Ras protein in the aqueous phase, while lovastatin did result in such an accumulation of Ras (Fig. 6).

DISCUSSION

We have shown that the monoterpene POH can inhibit type I and type II GGPTases in an NIH3T3 cell model.

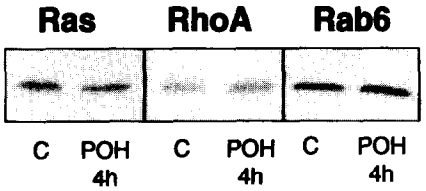

FIG. 5. Effect of POH on the levels of Ras, RhoA, and Rab6 proteins. Proteins from control and POH-treated cells were separated by SDS–PAGE and transferred to PVDF™ membrane. Western blots for Ras, RhoA, and Rab6 proteins were carried out as described under Materials and Methods. Four hours of POH (1 mM) treatment did not change the levels of these proteins in comparison to those in control cells.

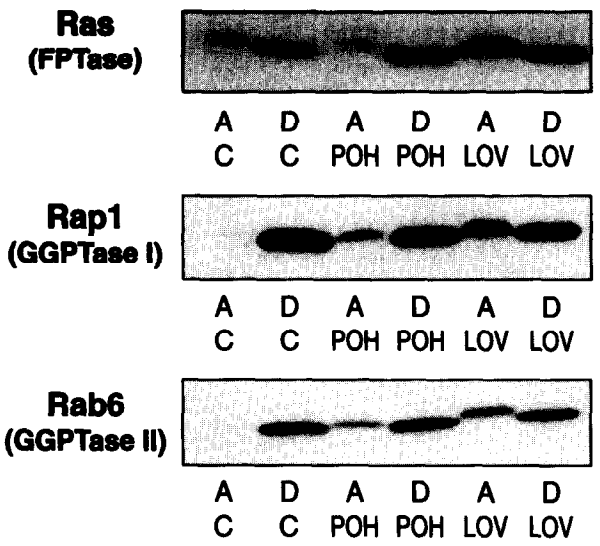

FIG. 6. Effect of POH on protein distribution between aqueous and detergent-enriched phases. Proteins from NIH3T3 cells were separated into aqueous and detergent-enriched phases, and Ras, Rap1, and Rab6 proteins from these two phases were analyzed by western blot as described under Materials and Methods. Key: A, aqueous phase; D, detergent-enriched phase; C, control cells; POH, 1 mM POH for 43 hr; and LOV, 30 μM lovastatin for 24 hr.

This inhibition resulted in an accumulation of the unprocessed forms of proteins that are geranylgeranylated by type I and type II GGPTases. While the IC_{50} values for the inhibition of type I and type II GGPTases are high, they are within the range of serum levels of monoterpenes observed when an anticancer dose of POH is administered for cancer treatment in rodents [2] and humans (Bailey HH and Gould MN, unpublished data).

The biochemical basis of POH-inhibited protein isoprenylation potentially could involve one or more enzymes in the mevalonate pathway. When protein prenylation inhibition is evaluated in cultured cells by assaying the incorporation of [^{14}C]mevalonolactone metabolic products into proteins, any observed inhibition could be due, at least in part, to the activity modulation of one or more enzymes in the pathway between mevalonate and the polyprenyl-protein transferases. For example, phenylacetate, an inhibitor of MVA-PP decarboxylase [26], has been shown to reduce the prenylated form of Ras [27]. Therefore, it is important to analyze the cellular concentrations of the isoprene intermediates between mevalonate and prenylated proteins in order to assess the relative contribution of the inhibition of polyprenyl-protein transferases and the potential modulation of the isoprene cosubstrates to changes in cellular protein prenylation. Of special concern are those prenylation inhibitors, including POH, that structurally mimic FPP or GGPP. Given the fact that most enzymes between mevalonate and GGPP interact with molecules that are structurally similar to FPP or GGPP, it is possible that such protein prenylation inhibitors could modulate one or more other enzymes in this pathway. We demonstrated that except for increasing FPP levels by approximately 50%, POH did not change the levels of other intermediates between mevalonate and isoprenylated proteins in NIH3T3 cells.

The reason for an accumulation of FPP despite the lack of inhibition of GGPP synthase or FPTase in POH-treated cells is unknown. Besides the inhibition of protein isoprenylation, POH can also inhibit cholesterol synthesis and CoQ synthesis [7]. The inhibition in the cholesterol synthesis pathway results in an accumulation of lathosterol, a precursor of cholesterol [7]. These specific modifications of lipid metabolism could potentially result in an accumulation of FPP. Importantly as discussed below, an increase in FPP of this magnitude did not modulate protein prenylation.

The data presented here demonstrate that POH inhibits type I and type II GGPTases in NIH3T3 cells. The isoprenylation of RhoA (substrate of type I GGPTase) and Rab6 (substrate of type II GGPTase) was inhibited by POH in *in vivo* [^{14}C]mevalonolactone-labeling studies. These observations were extended by demonstrating that the inhibition of isoprenylation of RhoA and Rab6 in NIH3T3 cells was due to the direct inhibition of the activities of type I and type II GGPTases and not to changes in cellular levels of GGPP, RhoA, and Rab6 protein during the terpene exposure time period. Cell lysate studies further confirmed the ability of POH to inhibit the activity of type I and type II GGPTases. These inhibitions are likely functional in that it was shown that the inhibition of the activity of these two enzymes by POH resulted in an accumulation of the unprocessed forms of Rap1 and Rab6 proteins in NIH3T3 cells.

Ras isoprenylation was not inhibited *in vivo* by POH in this study. This is in contrast to both the previously reported results that demonstrated a modest level of inhibition of Ras farnesylation in POH-treated cells [4] and the competitive inhibition of purified bovine brain and yeast FPTase by POH *in vitro* [6]. The possibility that the 50% increase in the FPP level in POH-treated cells in this study (Fig. 1) masks an inhibition of FPTase from being observed was ruled out. Ras isoprenylation levels were not changed even when the FPP level was increased by 75% (Fig. 3). The lack of accumulation of the unprocessed form of Ras in POH-treated cells is also consistent with the suggestion that FPTase is not inhibited by POH. Recently, Hohl and Lewis [20] also showed that FPTase was not inhibited by monoterpenes *in vivo*. They compared the relative amount of the farnesylated and unprocessed form of [^{35}S]methionine-labeled newly synthesized Ras protein and found that there was no difference in POH-treated cells and control cells [20]. The discrepancies between the *in vivo* and the *in vitro* inhibition of FPTase by FPTase inhibitors observed by us and other investigators [28–30] could be due to the inability of the drug to reach the target *in vivo*. Alternatively, the microenvironment for the enzymes might be

different, which could also cause the inconsistent inhibition of FPTase by POH from one cell line to another.

In summary, we have shown that the inhibition of protein prenylation in a 3T3 cell model is due directly to the inhibition of protein prenyltransferases. In this particular cell strain, this inhibition resulted specifically from the inhibition of GGPTase I and II and not FPTase. It is important to point out that the specifics of protein prenylation inhibition are modulated by cellular and/or environmental factors. This limits the ability to extrapolate from cell type to cell type or from an *in vitro* to *in vivo* environment. Recent studies in our laboratory have demonstrated the ability of chronic POH administration to inhibit protein isoprenylation in *in situ* rat mammary epithelial cells with the greatest inhibition involving type I GGPTase (Ren Z and Gould MN, unpublished data). Thus, monoterpenes have been shown to inhibit protein prenylation in many cell types in culture and at least in mammary epithelial cells *in situ* as well. The specifics of this inhibition vary among cell types and cellular environments. The key question that remains to be addressed is whether this monoterpene-induced biochemical activity is causal in the anticancer effects of this class of agents.

We thank Dr. Laurie Shepel for critical review of this manuscript. This work was supported by PHS NIH Grant R37-CA38128. Z. R. is the recipient of a traineeship from National Institutes of Health Training Grant 5T32-CA09471.

References

1. Mills JJ, Chari RS, Boyer IJ, Gould MN and Jirtle RL, Induction of apoptosis in liver tumors by monoterpene perillyl alcohol. *Cancer Res* **55:** 979–983, 1995.
2. Haag JD and Gould MN, Mammary carcinoma regression induced by perillyl alcohol, a hydroxylated analog of limonene. *Cancer Chemother Pharmacol* **34:** 477–483, 1994.
3. Stark MJ, Burke YD, McKinzie JH, Ayoubi AS and Crowell PL, Chemotherapy of pancreatic cancer with the monoterpene perillyl alcohol. *Cancer Lett* **96:** 15–21, 1995.
4. Crowell PL, Ren Z, Lin S, Vedejs E and Gould MN, Structure–activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation. *Biochem Pharmacol* **47:** 1405–1415, 1994.
5. Seachrist L, A fragrance for cancer treatment and prevention. *J NIH Res* **8:** 43, 1996.
6. Gelb MH, Tamanoi F, Yokoyama K, Ghomashchi F, Esson K and Gould MN, The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. *Cancer Lett* **91:** 169–175, 1995.
7. Ren Z and Gould MN, Inhibition of ubiquinone and cholesterol synthesis by the monoterpene perillyl alcohol. *Cancer Lett* **76:** 185–190, 1994.
8. Jirtle RL, Haag JD, Ariazi EA and Gould MN, Increased mannose 6-phosphate/insulin-like growth factor type II receptor and transforming growth factor β1 levels during monoterpene-induced regression of mammary tumors. *Cancer Res* **53:** 3849–3852, 1993.
9. Casey PJ and Seabra MC, Protein prenyltransferases. *J Biol Chem* **271:** 5289–5292, 1996.
10. Gibbs JB, Ras C-terminal processing enzymes—new drug targets? *Cell* **65:** 1–4, 1991.
11. Jackson JH, Cochrane CG, Bourne JR, Solski PA, Buss JE and Der CJ, Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. *Proc Natl Acad Sci USA* **87:** 3042–3046, 1990.
12. Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE and Der CJ, Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc Natl Acad Sci USA* **89:** 6403–6407, 1992.
13. Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, Giuliani EA, Gomez RP, Graham SL, Hamilton K, Handt LK, Hartman GD, Koblan KS, Karl AM, Miller PJ, Mosser SD, O'Neill TJ, Rands E, Schaber MD, Gibbs JB and Oliff A, Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nat Med* **1:** 792–797, 1995.
14. Kohl NE, Wilson FR, Mosser SD, Giuliani E, deSolms SJ, Conner MW, Anthony NJ, Holtz WJ, Gomez RP, Lee T-J, Smith RL, Graham SL, Hartman GD, Gibbs JB and Oliff A, Protein farnesyltransferase inhibitors block the growth of *ras*-dependent tumors in nude mice. *Proc Natl Acad Sci USA* **91:** 9141–9145, 1994.
15. Nagasu T, Yoshimatsu K, Rowell C, Lewis MD and Garcia AM, Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. *Cancer Res* **55:** 5310–5314, 1995.
16. Qiu R-G, Chen J, Kirn D, McCormick F and Symons M, An essential role for Rac in Ras transformation. *Nature* **374:** 457–459, 1995.
17. Qiu R-G, Chen J, McCormick F and Symons M, A role for Rho in Ras transformation. *Proc Natl Acad Sci USA* **92:** 11781–11785, 1995.
18. Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF and Der CJ, Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene* **10:** 2289–2296, 1995.
19. Crowell PL, Chang RR, Ren Z, Elson CE and Gould MN, Selective inhibition of isoprenylation of 21–26-kDa proteins by the anticarcinogen *d*-limonene and its metabolites. *J Biol Chem* **266:** 17679–17685, 1991.
20. Hohl RJ and Lewis K, Differential effects of monoterpenes and lovastatin on RAS processing. *J Biol Chem* **270:** 17508–17512, 1995.
21. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A, Monaghan R, Currie S, Stapley E, Albers-Schonberg G, Hensens O, Hirshfield J, Hoogsteen K, Liesch J and Springer J, Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc Natl Acad Sci USA* **77:** 3957–3961, 1980.
22. Watson JA, Harvel CM, Lobos DV, Baker FC and Morrow CJ, Isoprenoid synthesis in isolated embryonic Drosophila cells. Sterol-independent regulatory signal molecule is distal to isopentenyl 1-pyrophosphates. *J Biol Chem* **260:** 14083–14091, 1985.
23. Lutz RJ, McLain TM and Sinensky M, Feedback inhibition of polyisoprenyl pyrophosphate synthesis from mevalonate *in vitro*. Implications for protein prenylation. *J Biol Chem* **267:** 7983–7986, 1992.
24. Gutierrez L, Magee AI, Marshall CJ and Hancock JF, Post-translational processing of $p21^{ras}$ is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J* **8:** 1093–1098, 1989.
25. Garcia AM, Rowell C, Ackermann K, Kowalczyk JJ and Lewis MD, Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. *J Biol Chem* **268:** 18415–18418, 1993.
26. Castillo M, Martinez-Cayuela M, Zafra MF and Garcia-Peregrin E, Effect of phenylalanine derivatives on the main

regulatory enzymes of hepatic cholesterogenesis. *Mol Cell Biochem* **105:** 21–25, 1991.
27. Shack S, Chen L-C, Miller AC, Danesi R and Samid D, Increased susceptibility of *ras*-transformed cells to phenylacetate is associated with inhibition of $p21^{ras}$ isoprenylation and phenotypic reversion. *Int J Cancer* **63:** 124–129, 1995.
28. Gibbs JB, Pompliano DL, Mosser SD, Rands E, Lingham RB, Singh SB, Scolnick EM, Kohl NE and Oliff A, Selective inhibition of farnesyl-protein transferase blocks ras processing *in vivo*. *J Biol Chem* **268:** 7617–7620, 1993.
29. Kohl NE, Mosser SD, deSolms SJ, Giuliani EA, Pompliano DL, Graham SL, Smith RL, Scolnick EM, Oliff A and Gibbs JB, Selective inhibition of *ras*-dependent transformation by a farnesyltransferase inhibitor. *Science* **260:** 1934–1937, 1993.
30. James GL, Goldstein JL, Brown MS, Rawson TE, Somers TC, McDowell RS, Crowley CW, Lucas BK, Levinson AD and Marsters JC Jr, Benzodiazepine peptidomimetics: Potent inhibitors of Ras farnesylation in animal cells. *Science* **260:** 1937–1942, 1993.