Mammalian cells can use exogenous isoprenols to generate isoprenoid diphosphate substrates for protein isoprenylation, but the mechanism, efficiency, and biological importance of this process are not known. We developed mass spectrometry-based methods using chemical probes and newly synthesized stable isotope-labeled tracers to quantitate incorporation of exogenously provided farnesol, geranylgeraniol, and unnatural analogs of these isoprenols containing an aniline group into isoprenoid diphosphates and protein isoprenylcysteines by cultured human cancer cell lines. We found that at exogenous isoprenol concentrations >10 μM, this process can generate as much as 50% of the cellular isoprenoid diphosphate pool used for protein isoprenylation. Mutational activation of p53 in MDA-MB-231 breast cancer cells up-regulates the mevalonate pathway to promote tumor invasiveness. p53 silencing or pharmacological inhibition of HMG-CoA reductase in these cells decreases protein isoprenylation from endogenously synthesized isoprenoids but enhances the use of exogenous isoprenols for this purpose, indicating that this latter process is regulated independently of the mevalonate pathway. Our observations suggest unique opportunities for design of cancer cell-directed therapies and may provide insights into mechanisms underlying pleiotropic therapeutic benefits and unwanted side effects of mevalonate pathway inhibition.

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5 The abbreviations used are: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FOH, farnesol; GOGH, geranylglycerol diphosphate; AGH, anilinoglycerol diphosphate; AFPP, anilinofarnesol diphosphate; AG-Cys, anilinoglycylcysteine; F-Cys, farnesylcysteine; GG-Cys, geranylglycylcysteine; AFOH, anilinofarnesol.

Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are isoprenoid diphosphate intermediates in the mevalonate pathway. FPP and GGPP are formed by head-to-tail condensation of five-carbon isoprene units from isopentenyl diphosphate and dimethylallyl diphosphate to form geranyl diphosphate (GPP). Subsequent steps involve sequential conjugation of the isopentenyl group of isopentenyl diphosphate with GPP to form the 15-carbon FPP and the 20-carbon GGPP, respectively. FPP and GGPP are substrates for protein prenyltransferases that catalyze post-translational isoprenylation of cysteine residues at the C termini of a wide variety of proteins. Condensation of two molecules of FPP by squalene synthase initiates the formation of mevalonate pathway end products that include cholesterol, numerous steroid hormones, coenzyme Q10, and dolichol (1).

The mevalonate pathway produces FPP and GGPP without making the corresponding isoprenols. However, many cells can incorporate an exogenously provided unnatural analog of farnesol (FOH), anilinoglycerol (AGOH), into a diphosphate derivative that can then serve as a substrate for protein isoprenylation (2–6). Studies using radiolabeled FOH and geranylgeraniol (GGOH) suggest that these naturally occurring isoprenols are substrates for the same pathway (Fig. 1) (7–9). The relative efficiency and biochemical relationship of this “isoprenol-dependent,” “scavenging,” or “recycling” pathway to the much better characterized mevalonate pathway are not known. This information could be of fundamental importance to understanding the efficacy and side effects associated with pharmacological inhibition of the rate-limiting enzyme of the mevalonate pathway, HMG-CoA reductase, which is a widely
used clinical strategy for treatment of hypercholesterolemia and protection from coronary artery disease but may also have benefits in other settings (10). By depleting FPP and GGPP, statins also inhibit protein isoprenylation and synthesis of other mevalonate pathway end products. Inhibition of isoprenylation of Ras and Rho family small GTPases as substrates for prenylation of CAAX-sequence-containing proteins. Isoprenoid diphosphates derived from exogenously provided isoprenoids such as isotope-labeled or xenobiotic-containing analogs of FOH and GGOH can also serve as a source of isoprenoid precursors for protein prenylation. Homologation of FPP analogs to GGPP analogs occurs in cells using mevalonate pathway-derived isopentenyl diphosphate. FTase, farnesyltransferase; GGTase, geranylgeranyltransferase.

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**EXPERIMENTAL PROCEDURES**

**General Reagents**—Cell lines were purchased from American Type Culture Collection. High glucose DMEM, Dulbecco’s PBS, HEPES, penicillin/streptomycin, and RPMI 1640 medium were from Invitrogen. FBS was from Atlanta Biologicals, and protease from Streptomyces griseus (Pronase E) was from Sigma-Aldrich. HPLC-grade organic solvents (acetone, butyl alcohol, chloroform, isopropyl alcohol, methanol, and dimethyl sulfoxide (DMSO)) were from Fisher. Other chemicals and reagents, including octyl β-d-glucopyranoside, calcium acetate, doxycycline hyclate, insulin, chola toxin, DMEM/Ham’s nutrient mixture F-12, and hydrocortisone, were from Sigma-Aldrich. EGF was from PeproTech, and horse serum was from Invitrogen.

**Chemical Synthesis**—The compounds used in this study, including mass-labeled isoprenoid diphosphates and isoprenylcysteines, were synthesized as described previously (2, 6, 16–18).

**Cell Culture and Labeling**— Cultures of MDA-MB-231 and other cell lines used in this study were maintained in high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin. SUIT-2 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured at 10% confluence and incubated in a humidified atmosphere containing 5% CO₂ until fully confluent. MCF-10A cells were cultured in DMEM/Ham’s nutrient mixture F-12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml chola toxin, 10 µg/ml insulin, 100 units/ml penicillin, and 100 µg/ml streptomycin. For metabolic labeling experiments, cells were treated with various concentrations of isoprenols as described previously for analysis of AGOH incorporation into proteins (2). For studies of the effects of statins, cells were cultured in the presence or absence of GGOH-d₅₄ for 6 days with supplementation on day 3 with concurrent treatment with vehicle or mevinolin at concentrations of 1–25 µg/ml. Stably transfected MDA-MB-231 clonal cell lines with inducible p53-targeting engineered microRNAs were cultured in the presence or absence of doxycycline to induce p53 silencing as described (15).

**Extraction of Isoprenols and Isoprenoid Diphosphates**—Extraction of isoprenoid diphosphates was accomplished using the method previously described (19) with modifications as noted below. Cells cultured to confluence in the presence of vehicle control (0.03% DMSO) or vehicle containing isoprenols were washed once with PBS and lifted using trypsin/EDTA into 15-ml polypropylene tubes. Cells were washed once with ice-cold PBS and pelleted by centrifugation at 4 °C for 10 min at 20,000 × g. The cell pellet was resuspended in 1.2 ml of ice-cold freshly prepared extraction solvent (1:1.5:5 isopropyl alcohol, 75 mM ammonium hydroxide, and acetone) containing 1.0% octyl β-d-glucopyranoside. Anilinogeraniol diphosphate (AGPP)-d₅ or anilinofarnesol diphosphate (AFPP)-d₅ (500 pmol) was added as a recovery standard and for quantitation of all isoprenoid diphosphates recovered from cells. The mixture was vortexed for 5
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min, sonicated in a water bath containing ice for 5 min, and then centrifuged for 5 min at 20,000 × g to precipitate proteins. The supernatant was transferred to a new tube, and the protein pellet was re-extracted with 1.5 ml of extraction solvent and centrifuged for 5 min at 20,000 × g. The supernatants were combined and evaporated to dryness under a stream of nitrogen. The residue was resuspended in 100 μl of 25 mM ammonium hydroxide containing 1.0% octyl β-D-glucopyranoside, vortexed, and transferred to a polypropylene autosampler vial for HPLC-MS/MS analysis. FOH and GGOH were extracted from serum or human plasma using acetonitrile to precipitate the protein. The supernatant was transferred to a new tube, whereas the lower phase was re-extracted with 2 ml of water-saturated n-butyl alcohol. After centrifugation for 10 min at 1500 × g, the upper phase was harvested and concentrated to a final volume of 0.1 ml.

Quantitation of Isoprenols and Isoprenoid Diphosphates by Tandem Mass Spectrometry—LC-MS/MS analyses of isoprenoid phosphates were performed using an AB SCIEX 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer in multiple reaction monitoring mode. Isoprenoid phosphates were separated using a Macherey-Nagel NUCLEODUR C8 gravity column (5 μm, 125 × 2 mm). The mobile phase consisted of 25 mM ammonium acetate with 0.1% triethylamine as solvent A and acetonitrile and 25 mM ammonium acetate (4:1) with 0.1% triethylamine as solvent B. Separation was achieved using a linear gradient of 0–100% solvent B over 10 min. The flow rate was 0.5 ml/min with a column temperature of 30 °C (2). The sample injection volume was 10 μl. Unless noted otherwise, the mass spectrometer was operated in negative electrospray ionization mode. Optimal ion source settings determined by infusion of synthetic standards were a declustering potential of −70 V, entrance potential of 10 V, collision energy of −54 V, collision cell exit potential of −1 V, curtain gas of 20 p.s.i., ion spray voltage of −4500 V, ion source gas of 40 p.s.i., and temperature of 550 °C. Multiple reaction monitoring transitions were as follows: FPP-d6, m/z 387.6/78.8 and 387.3/158.5; GGPP-d6, m/z 455.9/78.8 and 455.9/158.6; AGPP, m/z 403.9/78.9 and 403.9/158.6; AFPP, m/z 472.8/79.0 and 472.8/158.4; AGPP-d6, m/z 408.9/78.9 and 408.9/158.6; GGPP, m/z 449/78.8 and 449/158.7; FPP, m/z 381/79 and 381/159; GPP, m/z 312.7/79 and 312.7/158.7; geranylgeranyl phosphate, m/z 369/79; geranyl phosphate, m/z 233/79; and anilinoaneriolip phosphat, m/z 323.9/78.9. AGPP was also detected in positive mode with instrument-optimized ion source settings by monitoring transitions m/z 406/228, 406/193, and 406/107. Peak identification and integration were accomplished using AB SCIEX Analyst software. When necessary, calibration was accomplished by reference to standard curves obtained using material that was independently quantitated by digestion and phosphorus determination. Isoprenoid phosphates were quantitated by stable isotope dilution using AGPP-d5 or AFPP-d5 as the internal standard. Isoprenols were measured using the chromatography method noted below for prenylcysteines by monitoring the following ion pairs: 205.3/81 and 205.3/121 for FOH, 211.2/81 and 211.3/121.6 for FOH-d6, 273.2/81 and 273.121.6 for GGOH, and 279.3/81.2 and 279.3/122.2 for GGOH-d6. The isoprenoid diphosphate assays have a lower limit of detection of 0.1 pmol and were linear up to column loadings of 100 pmol.

Preparation of Prenylcysteines from Cultured Cells—Prenylcysteines were prepared from protein pellets obtained after extraction of isoprenoid diphosphates from labeled cells. The protein fractions were resuspended in 50 mM HEPES (pH 7.4) and 2 mM calcium acetate in a bath sonicator. Small aliquots of the resuspended sample were used for protein concentration estimation by BCA. Known quantities of labeled proteins were digested by overnight incubation with Pronase E (10 mg) at 37 °C. Proteolysis was terminated by the addition of 2 ml of water-saturated n-butyl alcohol, anilinoaneriolipynylcysteine (AG-Cys)-d5, and anilinoaneriolipynylcysteine (AF-Cys)-d5 (50 pmol) added as a standard or for quantitation of the prenylcysteines, and the mixture was centrifuged at 1500 × g for 10 min to produce two phases. The upper phase was harvested into a new tube, whereas the lower phase was re-extracted with 2 ml of water-saturated n-butyl alcohol. After centrifugation for 10 min at 1500 × g, the upper phase was harvested and
The sample injection volume was 10 μl. The mass spectrometer was operated in the positive electrospray ionization mode with optimal ion source settings determined by synthetic standards of prenylcysteines with a declustering potential of 56 V, entrance potential of 10 V, collision energy of 25 V, collision cell exit potential of 4 V, curtain gas of 20 p.s.i., ion spray voltage of 5500 V, ion source gas 1/gas 2 of 40 p.s.i., and temperature of 550 °C. Multiple reaction monitoring transitions were as follows: m/z 349.2/135.1 and 349.2/228.1 for AG-Cys; m/z 326.3/95 and 326.3/81.3 for farnesylcysteine (F-Cys); m/z 394.3/122.2 and 394.3/81 for geranylgeranylcysteine (GG-Cys); m/z 417.3/203.1 and 417.3/93.1 for AF-Cys; and m/z 354.3/107.1 and 354.3/135.1 for AG-Cys-d₅; m/z 422.3/203.1 and 422.3/81.1 for AF-Cys-d₅; m/z 332.3/95 and 332.3/81.3 for F-Cys-d₆; and m/z 400.3/149.3, 400.3/122.2, and 400.3/81 for GG-Cys-d₆. Calibration was accomplished by reference to standard curves obtained using known quantities of synthetic prenylcysteine standards. These assays were linear up to column loadings of 100 pmol and had detection limits of 0.01 pmol.
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RESULTS

Incorporation of Exogenous Isoprenols into Isoprenoid Diphosphates by MDA-MB-231 Cells—FPP, GGPP, aniline-containing analogs, and deuterium-labeled variants of these compounds and their corresponding isoprenols shown in Fig. 2 were synthesized as described under “Experimental Procedures” and analyzed by electrospray ionization HPLC-MS/MS with quantitation accomplished by stable isotope dilution (Fig. 3, A and B, and supplemental Figs. S1 and S2). Incubation of MDA-MB-231 cells with exogenously supplied FOH-6d6 resulted in formation of FPP-6d6 and GGPP-6d6 (Fig. 3C). The synthetic isoprenol AGOH has been widely used as a probe to monitor protein isoprenylation in live cells by Western blotting using antibodies that recognize the aniline moiety (6). As previously reported in other cell types (2), incubation of MDA-MB-231 cells with AGOH resulted in accumulation of the corresponding diphosphate, AGPP, identifying the molecular intermediate in the process by which AGOH becomes a substrate for protein prenyltransferases (Fig. 3D). We concurrently discovered that cells also formed AFPP (see Fig. 2 for structure) from AGOH, which we presume is a result of condensation of AGPP with an additional isoprenol unit (Fig. 3D; see also Fig. 1). To investigate the efficiency of these processes, we incubated cells with increasing concentrations of the synthetic aniline-containing isoprenols and monitored their incorporation into isoprenoid diphosphates. We observed concentration-dependent formation of both AGPP and AFPP when cells were incubated with exogenous AGOH. We also found that exogenously provided anilinofernol (AFOH) was converted to AFPP. The levels of isoprenoid diphosphates formed from these exogenous unnatural isoprenol probes were comparable to the levels of endogenously formed FPP and GGPP, and levels of these endogenous isoprenoid diphosphates were similar to levels reported by other researchers using an enzymatic assay (20).

However, total cellular isoprenoid diphosphate levels increased additively in the presence of these unnatural exogenous isoprenols (Fig. 4A). Importantly, we also observed accumulation of FPP-6d6 and GGPP-6d6 in cells treated with increasing concentrations FOH-6d6, indicating that the ability of these cells to generate isoprenoid diphosphates from exogenous isoprenols is not limited to these aniline-containing analogs. The additive increases in isoprenoid diphosphates observed with the aniline-containing probes were also observed when cells were incubated with FOH-6d6 at concentrations in excess of 20 µM, suggesting that rather than being a consequence of AGPP accumulating because it cannot be further metabolized by the mevalonate pathway, exogenous isoprenols are metabolized to generate a pool of isoprenoid diphosphates that is regulated and accumulates separately from that generated by the mevalonate pathway (Fig. 4B). We used these methods to compare incorporation of AGOH and FOH-6d6 into isoprenoid diphosphates in three different cancer cell lines, MDA-MB-231 breast cancer cells, HepG2 hepatoma cells, and SUIT-2 pancreatic cancer cells. Formation of isoprenoid diphosphates from these exogenous precursors was 2–4-fold more efficient in MDA-MB-231 cells than in the two other cell types (Tables 1 and 2).

Efficient Use of Exogenous Isoprenols for Protein Isoprenylation in MDA-MB-231 Cells—To monitor and quantify the use of exogenous isoprenols to generate substrates for protein isoprenylation, we developed methods to analyze cysteine thiethioles of FOH, GGOH, and the aniline-containing isoprenol probes by electrospray ionization HPLC-MS/MS (supplemental Figs. S3 and S4). Quantitation was again accomplished using stable isotope dilution with mass-labeled internal standards (Fig. 5, A and B). The site-specific deuterium label enabled unequivocal assignment of the farnesyl fragmentation in the mass spectra of the isoprenoid cysteines. The proposed fragmentation scheme is shown in Fig. 5A. First, cleavage of the thiethiole occurs, giving either cysteine (m/z 122 for both molecules) or the farnesyl ion observed at m/z 205 (m/z 211 for FOH-6d6-containing analytes). Subsequent neutral loss of the terminal butene (−56, C4H8) for d6-FOH and C5H8 for d6-FOH-6d6) gives the 3,7-dimethyl-2,6,8-nonatrienyl ion at m/z 149 common to both molecules. Finally, neutral loss of isoprene (−68, C8H16) gives the 3-methyl-2,4-pentadienyl ion, which is the most intense fragment peak in both d6 and d6 spectra at m/z 81. A
TABLE 1
Levels of AGPP and AFPP formed in MDA-MB-231, HepG2, and SUIT-2 cells incubated with increasing concentrations of AGOH

Cells were incubated with the indicated concentrations of AGOH for 72 h. The levels of cell-associated AGPP and AFPP were determined as described under “Experimental Procedures.” The limit of detection determined using an offline calibration with AGPP and AGPP-d5 that were quantitated by accurate mass measurements was 0.1 pmol. Values noted as ND (not detected) were below this limit.

| Exogenous AGOH | MDA-MB-231 | HepG2 | SUIT-2 | MDA-MB-231 | HepG2 | SUIT-2 |
|----------------|------------|-------|--------|------------|-------|--------|
| 20 μM          | 0.4        | 0.1   | ND     | 0.2        | ND    | ND     |
| 40 μM          | 0.9        | 0.4   | ND     | 0.3        | ND    | ND     |
| 60 μM          | 1.3        | 0.5   | 0.1    | 0.4        | ND    | ND     |

TABLE 2
Levels of FPP-d6 and GGPP-d6 formed in MDA-MB-231, HepG2, and SUIT-2 cells incubated with increasing concentrations of FOH-d6

Cells were incubated with the indicated concentrations of FOH-d6, and the levels of cell-associated FPP-d6 and GGPP-d6 were determined as described under “Experimental Procedures.” The limit of detection determined using FPP, GGPP, and AGPP-d5 that were quantitated by accurate mass measurements was 0.1 pmol. Values noted as ND (not detected) were below this limit.

| Exogenous FOH-d6 | MDA-MB-231 | HepG2 | SUIT-2 | MDA-MB-231 | HepG2 | SUIT-2 |
|------------------|------------|-------|--------|------------|-------|--------|
| 20 μM            | 0.6        | 0.0   | ND     | 0.4        | ND    | ND     |
| 40 μM            | 1.9        | 0.6   | ND     | 0.9        | 0.1   | ND     |
| 60 μM            | 2.9        | 0.8   | ND     | 0.6        | ND    | ND     |

FIGURE 5. Quantitation of prenylcysteines in MDA-MB-231 cells treated with exogenous isoprenols. A, product ion spectrum of F-Cys-d6 in positive mode (see “Results” for interpretation). B, extracted ion chromatograms showing detection of prenylcysteine or prenylcysteine analogs. C, extracted ion chromatograms of F-Cys-d6 (m/z 332/81.3) and GG-Cys-d6 (m/z 400.3/122.2) from digests of total proteins obtained from MDA-MB-231 cells cultured with DMSO vehicle control (panel i) or 40 μM FOH-d6 (panel ii) for 72 h. D, extracted ion chromatograms of AG-Cys and AF-Cys from total protein extract digests from MDA-MB-231 cells treated with DMSO (panel i) or 40 μM AGOH (panel ii) for 72 h.
secondary fragmentation pathway involves cleavage of the thioether between the sulfur and the \( ^{-}\text{H}^\text{9252} -\text{H}^\text{9252}\) -carbon of cysteine, giving the ion at \( m/z 237 \) (\( d_6 \)) and \( m/z 243 \) (\( d_6 \)). The pattern of isotopomers for these fragment peaks is consistent with the presence of a sulfur atom. These methods were used to monitor incorporation of exogenous isoprenols into protein cysteine thioethers isolated after Pronase digestion of whole cell protein extracts. Consistent with our observations that exogenous isoprenols are converted to isoprenoid diphosphates by MDA-MB-231 cells (Figs. 3 and 4), we observed formation of F-Cys-\( d_6 \) and GG-Cys-\( d_6 \) in MDA-MB-231 cells treated with increasing concentrations of AGOH. C, the levels of F-Cys, GG-Cys, and GG-Cys-\( d_6 \) were measured in MDA-MB-231 cells incubated with vehicle or the indicated increasing concentrations of GGOH-\( d_6 \). D, the experiment shown in C was repeated in the presence of 1 \( \mu \text{M} \) mevinolin. Data in C and D are means ± S.D. (n = 2).

**FIGURE 6.** Effect of HMG-CoA reductase inhibition on levels of prenylcysteines in MDA-MB-231 cells incubated with increasing concentrations of exogenous isoprenols. A, F-Cys and F-Cys-\( d_6 \) levels in total protein digests from MDA-MB-231 cells incubated with increasing concentration of FOH-\( d_6 \). B, F-Cys and AG-Cys concentrations in total protein digests from MDA-MB-231 cells treated with increasing concentrations of AGOH. C, the levels of F-Cys, GG-Cys, and GG-Cys-\( d_6 \) were measured in MDA-MB-231 cells incubated with vehicle or the indicated increasing concentrations of GGOH-\( d_6 \). D, the experiment shown in C was repeated in the presence of 1 \( \mu \text{M} \) mevinolin. Data in C and D are means ± S.D. (n = 2).

exogenous AGOH into AG-Cys, and again consistent with our observation of AFPP in cells following incubation with AGOH, we observed formation of AF-Cys (Fig. 5D), indicating that AFPP is also an efficient prenyltransferase substrate. This observation is of potential relevance to the use of AGOH as a probe for monitoring protein isoprenylation in live cells by immunological detection of the protein-conjugated aniline moiety (6). We compared the concentration dependence with which exogenous FOH-\( d_6 \) or AGOH was incorporated into isoprenylcysteines in MDA-MB-231 cells. Formation of isoprenylcysteines from exogenous AGOH or FOH-\( d_6 \) appeared to occur at the expense of endogenously generated isoprenylcysteines.
because, unlike the additive accumulation of isoprenoid diphosphates produced from exogenous isoprenols shown in Fig. 3, the total level of isoprenylcysteines detected was unchanged in the presence of exogenous isoprenols. Incorporation of AGOH into AG-Cys could be observed at exogenous AGOH concentrations as low as 1 μM and was highly efficient, accounting for >50% of the total isoprenylcysteine pool, and this incorporation was apparently saturable at higher concentrations (Fig. 6, A and B, and Tables 3 and 4). Formation of isoprenylcysteines from endogenously formed substrates was decreased at exogenous isoprenol concentrations above 1 μM. The concentration dependence with which GGOH-d₆ was incorporated into GG-Cys by MDA-MB-231 cells was comparable to that observed for incorporation of FOH-d₆ into F-Cys (Fig. 6C). To explore the possibility that the increased ability of MDA-MB-231 cells to metabolize exogenous isoprenols was related to their highly transformed tumorigenic phenotype, we also examined incorporation of GGOH-d₆ into GG-Cys by MCF-10A cells, which are a non-tumorigenic breast epithelial cell line. Incorporation of GGOH-d₆ into GG-Cys was also detectable in these cells but was moderately reduced in comparison with MDA-MB-231 cells (Figs. 6C and 7A).

Metabolism of Exogenous Isoprenols to Generate Protein Prenyltransferase Substrates by MDA-MB-231 Cells Is Regulated Independently of the Mevalonate Pathway—We compared the effects of the HMG-CoA reductase inhibitor mevinolin on incorporation of GGOH-d₆ into GG-Cys by MDA-MB-231 and MCF-10A cells. A therapeutically relevant concentration of 1 μM mevinolin resulted in marked increases in incorporation of GGOH-d₆ into GG-Cys with a dramatic enhancement at the highest GGOH concentration examined (Fig. 6D). Similar observations were made with MCF-10A cells, although in the presence of 1 μM mevinolin, the magnitude of the enhancement of incorporation of GGOH-d₆ into GG-Cys was significantly lower than observed with MDA-MB-231 cells. A more substantial enhancement of the incorporation of GGOH-d₆ into GG-Cys was observed in the presence of a higher concentration (25 μM) of mevinolin (Fig. 7, B and C). Although the ability of cultured cells to use exogenous isoprenols to generate substrates for protein prenylation appears to be widespread, our finding that this process is relatively more efficient in MDA-MB-231 cells than in the other cell lines we examined is particularly interesting because up-regulation of mevalonate pathway genes resulting from an activating mutation in the p53 tumor suppressor promotes an invasive phenotype at least in part through mechanisms that involve geranylgeranylation of Rho family GTPases (15). We therefore compared the effects of p53 silencing or statin treatment on the incorporation of exogenous mass-labeled GGOH-d₆ into GG-Cys using a previously described MDA-MB-231 cell line with inducible transgenic expression of a p53-silencing engineered microRNA (15). Silencing of p53 expression by doxycycline was effective under our incubation conditions (Fig. 8A). We observed efficient incorporation of GGOH-d₆ into GG-Cys (~30% of unlabeled

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### TABLE 3

Levels of prenylcysteines derived from exogenous isoprenols in MDA-MB-231 and SUIT-2 cells

| Exogenous isoprenol | AGOH | FOH-d₆ |
|---------------------|------|-------|
|                     | AG-Cys | F-Cys | AF-Cys | GG-Cys | AF-Cys | GG-Cys |
|                     | MDA-MB-231 | SUIT-2 | MDA-MB-231 | SUIT-2 | MDA-MB-231 | SUIT-2 |
| 0.1 μM              | ND     | ND     | ND     | ND     | ND     | ND     |
| 1 μM                | 2.2    | ND     | ND     | ND     | ND     | ND     |
| 2.5 μM              | 10.3   | 1.6    | 2.6    | ND     | ND     | ND     |
| 5 μM                | 30.4   | 4.5    | 7.7    | ND     | ND     | ND     |
| 10 μM               | 59.4   | 3.6    | 13.5   | ND     | ND     | ND     |
| 20 μM               | 102.3  | 5.5    | 19.6   | ND     | ND     | ND     |
| 50 μM               | 84.6   | 12.5   | 11.3   | ND     | ND     | ND     |

### TABLE 4

Prenylcysteine levels in MDA-MB-231 cells treated with increasing concentrations of AGOH and FOH-d₆

| Exogenous isoprenol | AGOH | FOH-d₆ |
|---------------------|------|-------|
|                     | Total farnesylated | Total geranylgeranylated | Total farnesylated | Total geranylgeranylated |
|                     | AG-Cys | F-Cys | AF-Cys | GG-Cys | F-Cys | GG-Cys | F-Cys | GG-Cys |
| 0 μM                | 0     | 100   | 0     | 100   | 0     | 100   | 0     | 100   |
| 0.1 μM              | 0     | 100   | 0     | 100   | 0     | 100   | 0     | 100   |
| 1 μM                | 1     | 99    | 0     | 100   | 0     | 100   | 0     | 100   |
| 2.5 μM              | 6     | 94    | 0     | 100   | 0     | 100   | 0     | 100   |
| 5 μM                | 19    | 81    | 1     | 99    | 2     | 98    | 1     | 99    |
| 10 μM               | 36    | 64    | 2     | 98    | 6     | 94    | 5     | 95    |
| 20 μM               | 51    | 49    | 3     | 97    | 11    | 89    | 7     | 93    |
| 50 μM               | 41    | 59    | 1     | 99    | 10    | 90    | 7     | 93    |
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**FIGURE 7. Effect of HMG-CoA reductase inhibition on levels of prenylcysteines in MCF-10A cells incubated with increasing concentrations of exogenous GGOH.** A, the levels of F-Cys, GG-Cys, and GG-Cys-\(d_6\) were measured in MDA-MB-231 cells incubated with vehicle or the indicated increasing concentrations of GGOH-\(d_6\). The experiment shown in A was repeated in the presence of 1.0 \(\mu\)M mevinolin (B) or 25 \(\mu\)M mevinolin (C). Data are means ± S.D. (\(n = 2\)).

GG-Cys), although as noted above for studies using AGOH and FOH, incubation with exogenous GGOH also decreased total GG-Cys levels, suggestive of mevalonate pathway inhibition. Silencing p53 with doxycycline decreased GG-Cys levels to ~30% of the control, and this decrease was even more pronounced in the presence of GGOH. However, production of GG-Cys-\(d_6\) from exogenously provided GGOH-\(d_6\) was preserved following p53 silencing (Fig. 8B, right panel). This effect could be most clearly observed when the fractions of GG-Cys and GG-Cys-\(d_6\) were expressed as a percentage of total GG-Cys (Fig. 8B, left panel). This experiment was repeated using cells cultured in the presence of an HMG-CoA reductase inhibitor. Although formation of GG-Cys was decreased to ~10% of levels observed in the absence of HMG-CoA reductase inhibition, interestingly, HMG-CoA reductase inhibition resulted in increased incorporation of exogenous GGOH-\(d_6\) into GG-Cys-\(d_6\) relative to formation of GG-Cys from endogenous substrates, and this increase was further enhanced by p53 silencing. Taken together, these results indicate that the ability of MDA-MB-231 cells to generate GG-Cys from exogenous GGOH is controlled independently of components of the mevalonate pathway that are regulated by p53 or inhibited by statins.

**DISCUSSION**

Incorporation of exogenous isoprenols into substrates for protein isoprenylation and cholesterol synthesis has been observed in several mammalian cell types, although the mechanism and efficiency of this process in comparison with the well understood mevalonate pathway have been unclear. Our study addresses these issues by showing that exogenously supplied isoprenols, including FOH and GGOH, are converted to their diphosphate derivatives by several cultured human cancer cell lines. Isoprenoid diphosphates formed from exogenous isoprenols at concentrations in excess of 10 \(\mu\)M are effective substrates for protein prenyltransferases and can contribute significantly to the mass of protein isoprenylcysteines formed in three cultured cancer cell lines. Because formation of isoprenylcysteines from endogenous precursors is moderately suppressed in the presence of exogenous FOH and GGOH under conditions in which synthesis of isoprenoid diphosphates and isoprenylcysteines from these exogenous isoprenols is increased, it is possible that these two processes are regulated in a reciprocal manner. Our experiments using MDA-MB-231 cells support this idea further by showing a proportionate increase in use of exogenous isoprenols for protein prenylation under conditions in which the mevalonate pathway is suppressed by HMG-CoA reductase inhibition or p53 silencing.

The mechanism by which statins enhance metabolism of exogenous isoprenols is presently unclear, but the magnitude of these effects and our observations that these manipulations increase the levels of isoprenylcysteines beyond those present in untreated cells suggest that this does not involve simply decreasing competing pools of isoprenoid diphosphates that are normally produced by the mevalonate pathway. In preliminary studies, we also observed incorporation of exogenous mass-labeled FOH into cholesterol and coenzyme Q10 (data not shown). Although the efficiency of the incorporation of exogenous isoprenols into these downstream metabolites remains to be established, it appears likely that metabolism of isoprenoid diphosphates formed from exogenous isoprenols is not limited to their use as substrates for protein isoprenylation.

Our studies support the scheme shown in Fig. 1, in which isoprenols supplied exogenously or perhaps generated endogenously by degradation of isoprenylated proteins or dephosphorylation of isoprenoid diphosphates participate in a series of biochemical steps that operate in parallel to the mevalonate pathway to produce isoprenoid diphosphate substrates for protein prenylation. We also found that AGOH, which has been presumed to be a selective probe for protein farnesylation, can be converted to the GGPP analog AFPP, suggesting a point of intersection between these two pathways. Because we detected
formation of AF-Cys in cells treated with both AGOH and AFOH, it appears likely that immunological detection of incorporation of the aniline group of AGOH into proteins reports activities of both farnesyl- and geranylgeranyltransferases in live cells. The most parsimonious explanation for our observations is that cells contain kinases that convert exogenous isoprenols to their diphosphate derivatives. Although enzymes that can catalyze these reactions have been described in plants, their presence in mammalian cells has not been convincingly demonstrated (21). Direct interconversion of exogenous isoprenols and endogenous isoprenoid phosphates is another possibility, although, again, the necessary activities have not been reported in mammalian cells, and our observations suggest that, in at least two different cell lines, formation of isoprenoid phosphates from exogenous isoprenols is insensitive to and, at higher exogenous isoprenol concentrations, enhanced by mevalonate pathway inhibition or down-regulation.

We expect that the mass-labeled isoprenols reported here will prove to be useful for mass spectrometry-based approaches to enable unbiased identification of additional intermediates or end products of the metabolism of exogenous isoprenols by cultured mammalian cells.

Although our results demonstrate that the capacity of exogenous isoprenols to sustain protein isoprenylation is significant, the physiological importance of this process also remains to be established. Isoprenols, including FOH, are present in some plants and could therefore be a dietary source of precursors for protein isoprenylation. However, literature reports (22) indicate that levels of FOH in tissues are low in comparison with the concentrations needed to observe the phenomena reported in this study, and our own measurements of submicromolar levels of FOH and GGOH in bovine and horse sera and human plasma (data not shown) are consistent with these observations, suggesting that extracellular sources of isoprenols may not contribute significantly to isoprenoid diphosphate production at least in situations in which mevalonate pathway activity is high. However, our studies suggest that production of isoprenoid phosphates from FOH and GGOH is up-regulated and therefore might become important when the mevalonate pathway is inhibited.
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Although FOH and GGOH are not produced directly by the mevalonate pathway, they can be formed by degradation of isoprenylated proteins catalyzed by prenylcytoine lyases (23), so localized or cell-specific recycling of isoprenols may be a more important source of precursors for this pathway than circulating isoprenols. In particular, we and others have described phosphatase activities and identified at least one integral membrane enzyme that can dephosphorylate FPP and GGPP. In support of this idea, manipulation of the expression of this enzyme modulates protein isoprenylation in cultured cells (2, 24). Although a more expansive comparison of cells and tissues is clearly warranted, our study also suggests that there are significant differences in the efficiency with which different cultured mammalian cell lines metabolize exogenous isoprenols. Of a subset of human cancer cells examined, MDA-MB-231 cells incorporated exogenous isoprenols into isoprenoid diphosphates and isoprenylcysteines with the highest efficiency, although detectable metabolism of exogenous isoprenols was also observed with MCF-10A cells, which are a nonmalignant breast epithelial cell line, suggesting that the high activity of MDA-MB-231 cells is not simply a consequence of their transformed phenotype. Up-regulation of the mevalonate pathway in MDA-MB-231 cells resulting from gain-of-function mutations in the p53 tumor suppressor drives protein geranylgeranyl- and farnesylation-dependent invasion, suggesting that statins might be of benefit in the treatment of breast cancer or other cancers with similar p53 mutations. Our studies suggest that an increased capacity for metabolism of exogenous isoprenols may be a cellular response to mevalonate pathway inhibition by statins and that targeting this parallel pathway might be of additional therapeutic benefit or even provide new opportunities for therapeutic intervention. HMG-CoA reductase inhibition has been widely observed to have both beneficial actions and unwanted side effects in cardiovascular disease and other settings that are not directly related to attenuation of cholesterol synthesis. These effects have been speculated to involve inhibition of protein isoprenylation and production of other downstream metabolites, e.g. dolichol phosphate and coenzyme Q10, which are critical for protein glycosylation and mitochondrial function (25). Cell- and tissue-specific differences or inter-individual variation in the pathways for metabolism of exogenous isoprenols identified in this study might therefore be an important determinant of the efficacy or unwanted side effects of HMG-CoA reductase therapy.

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