Isoprenoids Influence Expression of Ras and Ras-Related Proteins†

Sarah A. Holstein,‡ Christine L. Wohlford-Lenane,§ and Raymond J. Hohl*,‡,§

Departments of Pharmacology and Internal Medicine, University of Iowa, Iowa City, Iowa 52242

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ABSTRACT: Mevalonate depletion by inhibition of hydroxymethylglutaryl coenzyme A reductase impairs post-translational processing of Ras and Ras-related proteins. We have previously shown that this mevalonate depletion also leads to the upregulation of Ras, Rap1a, RhoA, and RhoB. This upregulation may result from global inhibition of isoprenylation or depletion of key regulatory isoprenoid species. Studies utilizing specific isoprenoid pyrophosphates in mevalonate-depleted cells reveal that farnesyl pyrophosphate (FPP) restores Ras processing and prevents RhoB upregulation while geranylgeranyl pyrophosphate (GGPP) restores Rap1a processing and prevents RhoA and RhoB upregulation. Either FPP or GGPP completely prevents lovastatin-induced upregulation of RhoB mRNA. Inhibition of FPP or squalene synthase allowed for the further identification of the putative regulatory species. Studies involving the specific isoprenyl transferase inhibitors FTI-277 and GGTI-286 demonstrate that selective inhibition of protein isoprenylation does not mimic lovastatin's ability to increase Ras and RhoA synthesis, decrease Ras and RhoA degradation, increase RhoB mRNA, or increase total levels of Ras, Rap1a, RhoA, and RhoB. In aggregate, these findings reveal a novel role and mechanism for isoprenoids to influence levels of Ras and Ras-related proteins.

Post-translational modification of proteins containing the carboxyl-terminal CAAX consensus sequence by covalent linkage of the cysteine to isoprenyl moieties (i.e., isoprenylation) is central to intracellular localization and proper function of many regulatory proteins such as Ras and other members of its superfamily (*1*, *2*). The isoprenyl substrates for these reactions are either the 15-carbon farnesyl or the 20-carbon geranylgeranyl pyrophosphates that are derived from mevalonate (Figure 1). These isoprenoids are common to all eukaryotic cells and are required for protein isoprenylation. Depletion of mevalonate by HMG-CoA[1] reductase inhibition generally results in accumulation of unmodified proteins, likely a consequence of now-limited FPP and GGPP availability (*1*, *3*). This limitation reduces the covalent linkage of isoprenoid to protein catalyzed by the enzyme FPTase or GGPTase. Importantly, the unprenylated forms of Ras and Ras-related proteins may maintain partial function (*4*, *5*) and interfere with the activity of the isoprenylated proteins (*6*, *7*).

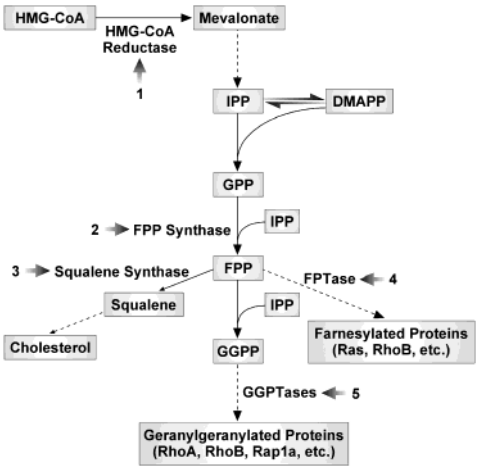

FIGURE 1: Isoprenoid biosynthetic pathway. Arrows indicate the sites of action targeted by the inhibitors used in these studies: target 1, lovastatin; target 2, pamidronate; target 3, zaragozic acid; target 4, FTI; target 5, GGTI.

Little is known of the regulatory influence that these mevalonate-derived isoprenoids exert on expression of isoprenylated proteins. We have demonstrated that depletion of mevalonate induces the upregulation of Ras and Ras-related proteins through transcriptional, translational, and post-translational processes (*8*). Given the role these proteins play in pathways regulating cell survival, proliferation, differentiation, and cytoskeletal organization (*9*, *10*), it seems likely that altered expression will manifest as markedly abnormal function. Furthermore, it has been suggested that total levels of Ras may be an important determinant of cell growth regardless of intracellular localization (*11*). Thus, it is important to better define the mechanisms for the upregulation that is observed with mevalonate depletion. Avail-

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* To whom correspondence should be addressed at the Department of Internal Medicine, C32 GH, University of Iowa, Iowa City, IA 52242. Phone: (319) 356-8110. Fax: (319) 353-8383. E-mail: raymond-hohl@uiowa.edu.

‡ Department of Pharmacology.

§ Department of Internal Medicine.

[1] Abbreviations: HMG-CoA, hydroxymethylglutaryl coenzyme A.; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; FTI, farnesyl transferase inhibitor; GGTI, geranylgeranyl transferase inhibitor, RIPA, radioimmune precipitation buffer.

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ability of selective FPTase and GGPTase inhibitors allows for evaluation of the role of FPP and GGPP to contribute to this upregulation. We present studies that distinguish between the upregulation induced by HMG-CoA reductase inhibition (Figure 1, target 1) and the effects of inhibition of FPTase and/or GGPTase (Figure 1, targets 4 and 5). Furthermore, these studies implicate that intermediates in the isoprenoid biosynthetic pathway play a role in regulating the expression of Ras and Ras-related proteins.

EXPERIMENTAL PROCEDURES

Cell Cultures and Reagents. The K562 cell line was purchased from the American Type Culture Collection (Manassas, VA). The K562 cell line is a human erythroleukemia line that was established from a patient with chronic myelogenous leukemia (*12*). K562 cells were grown in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, amphotericin (2.5 μg/mL), and glutamine (2 mM). Cells were grown at 37 °C and 5% CO_2 in T-75 culture flasks. Anti-RhoA, anti-RhoB, anti-Rap1a (specific for unmodified Rap1a (*13*); catalog number sc-1482), anti-β-tubulin, and anti-goat IgG HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NCC-004 anti-pan RAS antibody (*14*) was kindly provided by Dr. Setsuo Hirohashi (National Cancer Center, Tokyo). Anti-v-H-Ras (Ab-1, pan Ras) agarose conjugate was obtained from Calbiochem (San Diego, CA). Anti-mouse and anti-rabbit HRP-linked antibodies were obtained from Amersham (Piscataway, NJ). [^{35}S]-Express Protein Labeling Mix was purchased from Perkin-Elmer (Boston, MA). Methionine- and cystine-deficient RPMI medium was obtained from ICN (Costa Mesa, CA). The farnesyl transferase inhibitor FTI-277 and the geranylgeranyl transferase I inhibitor GGTI-286 were purchased from Calbiochem. Pamidronate was obtained from Novartis (East Hanover, NJ). DL-Mevalonic acid lactone (Sigma) was converted to mevalonate prior to use. Lovastatin, IPP, DMAPP, GPP, FPP, GGPP, squalene, cycloheximide, actinomycin D, and zaragozic acid A were purchased from Sigma.

Western Blot Analysis. Cells (1×10^6 cells/mL) were incubated with lovastatin, pamidronate, zaragozic acid, and various isoprenoid species for 24 h. For time course studies, cells were incubated with FTI and/or GGTI for 0−24 h with and without 1 h of pretreatment with actinomycin D or cycloheximide. Cells were then collected at 4 h intervals, washed with PBS, and lysed in RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton (v/v) X-100, 0.05 M Tris·HCl) containing protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin) and sodium orthovanadate. Protein content was determined using the Lowry method (*15*). Equivalent amounts of cell lysate were resolved by SDS−PAGE, transferred to poly(vinylidene difluoride) membrane, and probed with the appropriate antibodies. Blots probed for Ras, RhoA, RhoB, Rap1a, and β-tubulin were detected with HRP-linked secondary antibodies and ECL Western blotting reagents (Amersham Biosciences, Inc.) according to the manufacturer's protocols.

[^{35}S]Methionine Experiments. For pulse experiments, cells were incubated with or without FTI and/or GGTI for 4 or 24 h and were pulsed with [^{35}S]methionine during the last 4 h of each incubation. For pulse−chase experiments, cells were preincubated in methionine- and cystine-free RPMI medium with 2% FCS for 1 h and then pulsed with [^{35}S]-methionine (120 μCi/mL) for 4 h. Cells were then washed with complete RPMI medium plus 10 mM methionine, 3 mM cysteine, and 10% FCS and incubated for 0−24 h in the presence or absence of FTI and/or GGTI. Cells were lysed in RIPA buffer, and following preclearing, 200 μg of whole cell lysate was diluted in RIPA + 1% bovine serum albumin and incubated with agarose-conjugated antibodies at 4 °C. Ras immunocomplexes were obtained per the manufacturer's protocols. Immunocomplexes were washed with RIPA + 1% bovine serum albumin and 1X PBS + 1% bovine serum albumin. The pellets were fractionated by SDS−PAGE, and dried gels were exposed to film at −70 °C. Radioactivity of excised bands was determined using liquid scintillation counting.

Preparation and Synthesis of RNA Probe Templates. All RNA probes (with the exception of β-actin) used in this study were generated in our laboratory using the reverse transcriptase−polymerase chain reaction with the addition of an RNA polymerase site (Lig'nScribe, Ambion, Austin, TX) to the RT-PCR product. The human β-actin probe template was purchased from Ambion. A 300 bp human RhoA cDNA was generated using the following primer sets: CTC CGT CGG TTC TCT CGT TA and CCC ACA AAG CCA ACT CTA CC. The sequences for RhoB and *N*-Ras probes were previously described (*8*). An additional 60 bp is added to all the above templates when the T7 RNA polymerase site is added to the cDNA using the Lig'nScribe RNA Polymerase Promoter Addition Kit (Ambion). Each probe template was sequenced using an ABI Prism genetic analyzer (Perkin-Elmer Life Sciences) prior to use.

Preparation of RNA and Northern Blot Analysis. Total RNA was isolated from cells using the single-step method (*16*), lysing the cells in 1.2 mL of Trizol reagent (Invitrogen). Chloroform was added, the total RNA precipitated from the aqueous phase by the addition of isopropyl alcohol, the RNA pellet washed with ethanol and solubilized in RNase free water. The yield and purity of the total RNA were quantitated by measuring the ratio of the absorbances at 260 and 280 nm. RNA integrity was determined by examining the 28 and 18s rRNA bands on a 1.2% agarose, 2.2 M formaldehyde gel. Total RNA (20 μg) was separated on a 1.2% agarose, 2.2 M formaldehyde gel, transferred to a Hybond-N+ (Amersham) membrane by capillary action overnight, and UV-cross-linked. Northern blots were performed as described previously (*8*). To compensate for RNA gel loading artifacts, all blots were probed with ^{32}P-labeled human β-actin and ratios adjusted accordingly.

RESULTS

Importance of Isoprenoid Depletion on Upregulation of Ras and Ras-Related Proteins. There are increased amounts of total Ras, Rap1a, RhoA, and RhoB in lovastatin-treated cells as compared to control K562 cells (Figure 2). To determine whether this upregulation is due to the depletion of a specific isoprenoid species, K562 cells were coincubated with lovastatin and the isoprenoid pathway intermediates mevalonate, IPP, DMAPP, GPP, FPP, GGPP, and squalene. High concentrations of mevalonate (5 mM) completely

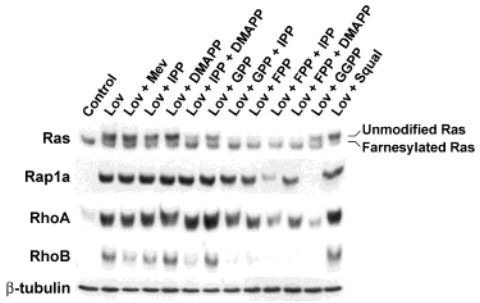

FIGURE 2: Effects of isoprenoid biosynthetic pathway intermediates on lovastatin-induced upregulation of Ras and Ras-related proteins. K562 cells were incubated for 24 h in the presence of 10 μM lovastatin (*Lov*), 30 μM mevalonate (*Mev*), 30 μM IPP, 30 μM DMAPP, 15 μM IPP + 15 μM DMAPP, 10 μM GPP, 10 μM GPP + 30 μM IPP, 10 μM FPP, 10 μM FPP + 30 μM IPP, 10 μM FPP + 30 μM DMAPP, 10 μM GGPP, or 10 μM squalene (*Squal*). These immunoblots were developed as described in the Experimental Procedures. Each lane contains an equivalent amount of protein from the cell lysate. The blots are representative of three independent experiments.

blocked lovastatin's effects (data not shown). Interestingly, low concentrations of mevalonate (30 μM) displayed differential effects in mitigating lovastatin-induced protein upregulation. A 30 μM concentration of mevalonate neither restored Ras or Rap1a processing nor prevented RhoA upregulation but did partially block upregulation of RhoB. Similarly, coincubation of lovastatin and IPP ± DMAPP did not alter levels of Ras, unmodified Rap1a, or RhoA but did partially prevent upregulation of RhoB. Biosynthesis of FPP requires both GPP and IPP; thus, while coincubation with GPP alone did not restore lovastatin-induced impairment of Ras processing, combinations of GPP and IPP were effective. Furthermore, coincubation with GPP plus IPP, but not GPP alone, completely blocked the upregulation of RhoB. FPP and GGPP also individually prevented the upregulation of RhoB. As predicted, cotreatment with FPP, but not GGPP, restored the farnesylation of Ras and prevented the overall increase of Ras protein levels. The geranylgeranylation of Rap1a was restored completely by GGPP and incompletely by the combination of FPP and IPP. Upregulation of RhoA was more efficiently prevented by GGPP than by FPP. Coincubation with squalene did not restore processing of Ras or Rap1a. Significantly, coincubation with squalene also did not prevent lovastatin-induced upregulation of RhoA and RhoB. In control experiments without lovastatin, none of the isoprenoids altered Ras or Ras-related protein levels (data not shown).

To further define the critical isoprenoids that regulate the expression of Ras and Ras-related proteins, additional experiments were performed in the presence of pamidronate. Pamidronate is a member of the bisphosphonate class of drugs that inhibits FPP synthase (Figure 1, target 2) (*17*, *18*). While the combination of GPP and IPP prevented upregulation of RhoB induced by lovastatin (Figure 2), it did not prevent upregulation in cells treated with both lovastatin and pamidronate (Figure 3A), consistent with the requirement for FPP synthesis. Likewise, the combination of GPP and IPP did not restore Ras processing in cells treated with both lovastatin and pamidronate. FPP restored Ras farnesylation and GGPP restored Rap1a geranylgeranylation in lovastatin/pamidronate-treated cells. That the combination of FPP and IPP was not as effective in restoring Rap1a processing in

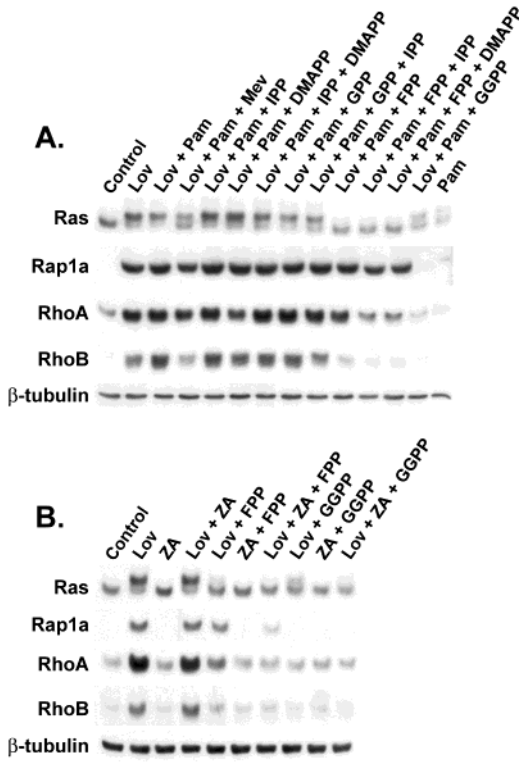

FIGURE 3: Effects of isoprenoid pyrophosphates in lovastatin- and pamidronate- or zaragozic acid-treated cells. (A) K562 cells were incubated for 24 h in the presence of 10 μM lovastatin (*Lov*), 100 μM pamidronate (*Pam*), 30 μM mevalonate (*Mev*), 30 μM IPP, 30 μM DMAPP, 15 μM IPP + 15 μM DMAPP, 10 μM GPP, 10 μM GPP + 30 μM IPP, 10 μM FPP, 10 μM FPP + 30 μM IPP, 10 μM FPP + 30 μM DMAPP, or 10 μM GGPP. (B) Cells were incubated for 24 h in the presence of 10 μM lovastatin (*Lov*), 100 μM zaragozic acid (*ZA*), 10 μM FPP, or 10 μM GGPP. These immunoblots were developed as described in the Experimental Procedures. Each lane contains an equivalent amount of protein from the cell lysate. The blots are representative of two independent experiments.

lovastatin/pamidronate-treated cells as compared to cells treated only with lovastatin may be due to the ability of pamidronate to serve as an inhibitor of GGPP synthase (*19*).

To further investigate the relative contributions of sterol and nonsterol species, the effects of the squalene synthase inhibitor zaragozic acid A (Figure 1, target 3) (*20*) were investigated. As shown in Figure 3B, incubation with zaragozic acid alone did not alter Ras or Rap1a processing and did not alter levels of RhoA or RhoB, providing further evidence for the role of nonsterol isoprenoid species. In addition, zaragozic acid did not alter lovastatin's effects. Interestingly, the addition of zaragozic acid to lovastatin-treated cells increased the efficiency of exogenous FPP and GGPP in restoring processing and preventing lovastatin-induced upregulation.

To delineate the mechanisms for isoprenoid prevention of lovastatin-induced upregulation of RhoB, analysis of RhoB mRNA was performed. As shown in Figure 4, the lovastatin-induced increase in RhoB mRNA was completely prevented by coincubation with mevalonate, FPP, or GGPP. Parallel experiments demonstrated that lovastatin did not alter RhoA or *N*-Ras mRNA levels.

Differential Effects of Isoprenoid Depletion and Isoprenyl Transferase Inhibition. Lovastatin increases de novo synthesis and decreases the degradation of Ras and RhoA (*8*).

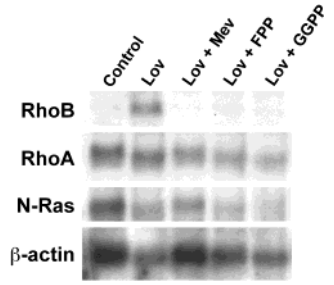

FIGURE 4: FPP and GGPP can prevent lovastatin-induced upregulation of RhoB mRNA. K562 cells were incubated for 24 h in the presence of 10 μM lovastatin (*Lov*), 5 mM mevalonate (Mev), 10 μM FPP, and 10 μM GGPP. Total RNA was isolated, and Northern blots were performed as described in the Experimental Procedures using RhoB-, RhoA-, or *N*-Ras-specific riboprobes. β-Actin levels are shown as a control. The blots are representative of two independent experiments.

To determine whether these observations result from only a reduction in isoprenylation or also require isoprenoid depletion, studies using inhibitors of isoprenyl transferases were performed. In striking contrast to the lovastatin data, treatment with FTI and/or GGTI did not significantly increase levels of newly synthesized Ras (Figure 5A). Similar results were found with RhoA (data not shown). Pulse−chase experiments were also performed (Figure 5B). The amount of labeled Ras declined over 24 h, and this decline was not altered by treatment with FTI and/or GGTI. Furthermore, the half-life of RhoA was not affected by incubation with FTI and/or GGTI (data not shown). Thus, for Ras and RhoA, inhibition of isoprenyl transferases does not alter protein synthesis or degradation in contrast to inhibition of HMG-CoA reductase. Control experiments demonstrated that treatment with FTI and/or GGTI did not alter the de novo synthesis or degradation of actin, had no effect on the incorporation of [^{35}S]methionine into total cellular protein pools, and did not alter the loss of ^{35}S from total protein pools over time as determined by trichloroacetic acid precipitation assays (data not shown).

We have previously shown that while depletion of mevalonate does not alter Ras mRNA levels, it does induce a significant increase in RhoB mRNA (*8*). Studies were performed to determine whether this pattern was also observed with the FTI and/or GGTI. Figure 6 demonstrates that treatment with FTI and/or GGTI for 4−24 h did not alter levels of *N*-Ras, RhoA, or RhoB mRNA. Levels of mRNA from cells treated with lovastatin for 24 h are shown as a control. Thus, isoprenyl transferase inhibitors do not upregulate RhoB as does mevalonate depletion.

Time course experiments were performed to determine whether levels of Ras, Rap1a, RhoA, and RhoB are affected by FTI and/or GGTI. As shown in Figure 7A, treatment with FTI or FTI/GGTI results in detection of unmodified Ras. Despite the presence of both modified and unmodified Ras, the total levels of Ras at 24 h do not increase to that observed with lovastatin. In addition, unmodified Rap1a is not detectable with FTI treatment but is present in cells treated with GGTI, although at lower levels than in lovastatin-treated cells. Significantly, the isoprenyl transferase inhibitors did not alter levels of RhoA and RhoB. This is in contrast to the marked upregulation of RhoA and RhoB induced by lovastatin. As a control and to allow for more direct comparison to the effects previously observed with lovastatin

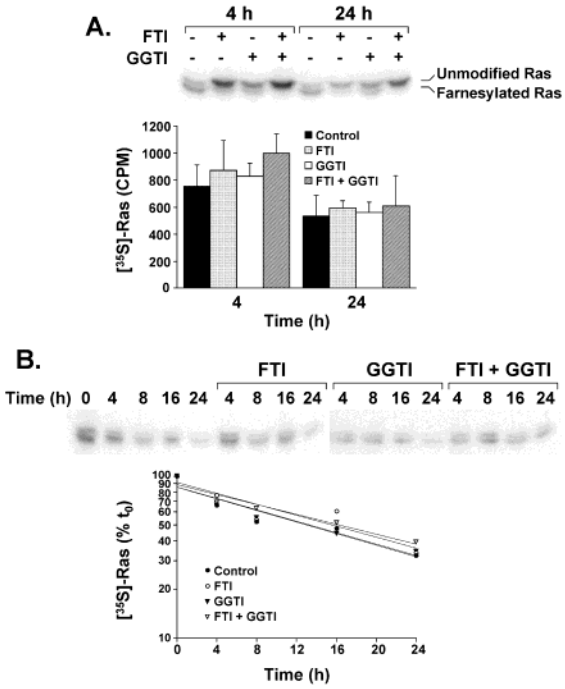

FIGURE 5: FTI and/or GGTI do not alter de novo synthesis or degradation of Ras. (A) K562 cells were incubated with or without FTI (100 nM) and/or GGTI (2 μM) for 4 and 24 h. Cells were pulsed with [^{35}S]methionine (120 μCi/10 $\times$ 10^6 cells) during the last 4 h of each incubation. Ras was immunoprecipitated, fractionated on SDS−PAGE, and exposed to film at −70 °C for 3 days. A representative gel is displayed. Bands were subsequently excised and radiolabel counted via liquid scintillation counting. The counts from three independent experiments are displayed in bar graph format as the mean ± SD (n = 3). Significant differences between cells incubated without or with isoprenyl transferase inhibitors were assessed using Student's t test, and P values were found to be >0.05. (B) Cells were pulsed with [^{35}S]methionine (120 μCi/mL) for 4 h and then chased in the absence or presence of FTI (100 nM) and/or GGTI (2 μM). Ras was immunoprecipitated, fractionated on SDS−PAGE, and exposed to film at −70 °C for 3 days. Bands were subsequently excised and counted via liquid scintillation counting. The counts are expressed as a percentage of the radioactivity at the conclusion of the pulse and are displayed in a semilog plot. The results are representative of two independent experiments.

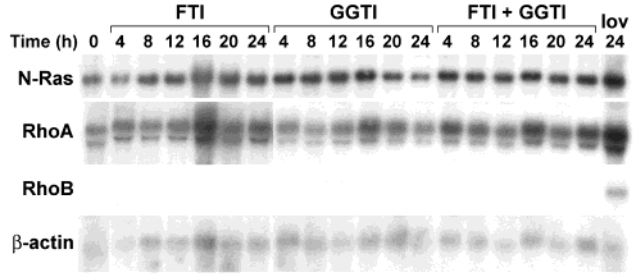

FIGURE 6: Effects of FTI and/or GGTI on *N*-Ras, RhoA, and RhoB mRNA levels. K562 cells were incubated with 100 nM FTI and/or 2 μM GGTI for up to 24 h or with 10 μM lovastatin for 24 h. Total RNA was isolated, and Northern blot analysis was performed with *N*-Ras-, RhoA-, or RhoB-specific riboprobes as described in the Experimental Procedures. β-Actin mRNA levels are shown as a control.

(*8*), cells were also pretreated with the protein synthesis inhibitor cycloheximide. This effectively blocked the appearance of unmodified Ras and Rap1a (Figure 7B). Confirming the finding that *N*-Ras mRNA levels are not altered by isoprenyl transferase inhibitors (Figure 6), pretreatment

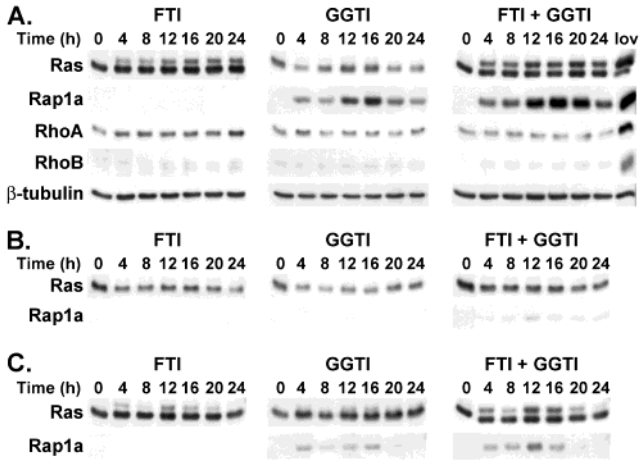

FIGURE 7: Effects of FTI and/or GGTI on Ras and Ras-related protein levels. (A) K562 cells were incubated with FTI (100 nM) and/or GGTI (2 μM) for up to 24 h, with cells collected every 4 h. (B) Cells were pretreated with cycloheximide (1.4 μg/ mL) for 1 h prior to addition of FTI and/or GGTI. (C) Cells were pretreated with actinomycin D (0.5 μg/mL) for 1 h prior to addition of FTI and/or GGTI. These immunoblots were developed as described in the Experimental Procedures. Each lane contains an equivalent amount of protein from the cell lysate. The blots reflect one study that is representative of two to four independent experiments.

with the transcription inhibitor actinomycin D only minimally affected the accumulation of unmodified Ras and Rap1a induced by treatment with FTI and GGTI (Figure 7C). Thus, on the basis of the data from this experiment and those depicted in Figures 5 and 6, treatment with FTI and GGTI, like lovastatin, results in the inhibition of isoprenylation, but unlike lovastatin, not the upregulation of Ras and Ras-related proteins.

Although we have shown that inhibition of isoprenyl transferases does not result in upregulation of Ras-related proteins and that FPP and GGPP can prevent lovastatin-induced upregulation, the possibility remained that FPP or GGPP restores the isoprenylation of a protein that is responsible for regulating the levels of the Ras-related proteins. Therefore, the ability of FPP and GGPP to prevent lovastatin-induced upregulation of RhoA and RhoB in the presence of isoprenyl transferase inhibitors was examined. As shown in Figure 8, the combination of FTI and GGTI resulted in the appearance of unmodified Ras and Rap1a. Treatment with FPP and GGPP did not restore Ras or Rap1a processing in the presence of FTI/GGTI. This was an expected result since the FTI and GGTI used in these studies are peptidomimetics and thus inhibit FPTase and GGPTase with respect to the CAAX-containing substrates (*21*, *22*). Addition of lovastatin to FTI/GGTI further increased the levels of unmodified Ras and Rap1a. Coincubation with FPP and GGPP in lovastatin/FTI/GGTI-treated cells restored levels of unmodified Ras and Rap1a to those in cells treated only with FTI/GGTI, thus demonstrating the ability of FPP and GGPP to prevent upregulation of Ras and Rap1a in the presence of continued inhibition of isoprenylation. Furthermore, lovastatin's upregulation of RhoA and RhoB was also prevented by coincubation with either FPP or GGPP in the presence of FTI/GGTI. These findings are additional evidence that the lovastatin-induced upregulation of these proteins is a consequence of isoprenoid, rather than isoprenylated protein, depletion.

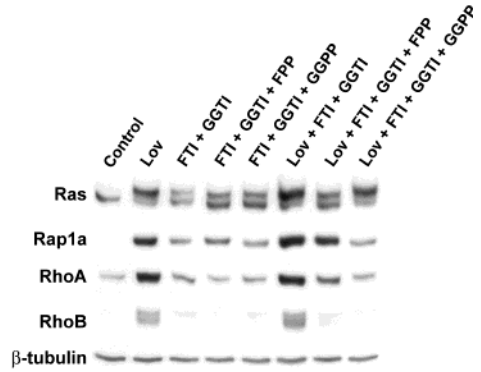

FIGURE 8: FPP and GGPP prevent mevalonate depletion-induced upregulation of RhoA and RhoB in the presence of isoprenyl transferase inhibitors. Cells were incubated with 10 μM lovastatin (*lov*), 10 μM FPP, 10 μM GGPP, 100 nM FTI, and 2 μM GGTI for 24 h. These immunoblots were developed as described in the Experimental Procedures. Each lane contains an equivalent amount of protein from the cell lysate. The blots are representative of two independent experiments.

DISCUSSION

We have previously demonstrated that lovastatin induces upregulation of Ras and Ras-related proteins (*8*). This upregulation results from accumulation of both unprenylated and isoprenylated species. Given the importance of the signaling pathways governed by these isoprenylated proteins and the recent evidence describing functions for unprenylated forms of these proteins, the precise mechanism(s) for this upregulation needs to be delineated. Lovastatin depletes cells of all of the mevalonate-derived isoprenoids (Figure 1). Our current studies examine whether the upregulation is a consequence of inhibition of isoprenylation or is due to depletion of specific regulatory isoprenoid pyrophosphates.

Impairment of protein isoprenylation by mevalonate depletion can be selectively restored by the addition of downstream isoprenoid pyrophosphates (Figure 1): FPP restores Ras processing, while GGPP restores Rap1a processing (Figure 2). Mevalonate depletion not only induces upregulation of these proteins, but also upregulates RhoA and RhoB (Figure 2). Our findings demonstrate that these specific isoprenoids, FPP and GGPP, can also impair lovastatin-induced upregulation. FPP prevents lovastatin-induced RhoB upregulation and partially prevents RhoA upregulation, while GGPP completely prevents upregulation of both RhoA and RhoB. Furthermore, we have shown that these isoprenoid pyrophosphates influence protein levels at multiple regulatory levels: the upregulation of RhoA, mediated through translational and transcriptional events, is prevented by GGPP (Figure 2), while both FPP and GGPP prevent lovastatin-induced transcriptional upregulation of RhoB mRNA (Figure 4).

Mechanisms for transcriptional upregulation may result from sterol- or specific isoprene-mediated effects. The former process has been well-characterized and described as involving sterols, sterol regulatory elements (SREs), and SRE binding proteins (SREBPs) (*23*). For Ras-related proteins our experiments suggest sterol-independent regulatory mechanisms. Figure 2 demonstrates that squalene does not abrogate lovastatin-induced upregulation of RhoA and RhoB. Squalene restores sterol synthesis but not isoprenoid pyrophosphate availability under conditions of mevalonate depletion (Figure

1). In addition, use of a squalene synthase inhibitor demonstrated that inhibition of de novo sterol synthesis does not alter the regulation of Ras and Ras-related proteins (Figure 3B). Furthermore, GGPP, which is not a precursor for sterol synthesis, prevents lovastatin-induced upregulation of RhoA and RhoB at both transcriptional (RhoB) and post-transcriptional (RhoA) levels. With regard to the former, GGPP has also been shown to transcriptionally regulate the expression of the ATP-binding cassette transporter A1 (ABCA1) (*24*).

Heretofore, FPP has not been shown to regulate transcriptional events. The combinations of IPP + DMAPP, GPP +IPP, FPP ± IPP or DMAPP, and GGPP all prevented lovastatin-induced upregulation of RhoB (Figure 2). Since GPP alone did not prevent this upregulation, the responsible isoprenoids were inferred to be at or below the level of FPP. This is further supported by the effect of the FPP synthase inhibitor pamidronate to block the effects of IPP + DMAPP or GPP + IPP (Figure 3A). Finally, Figure 4 clearly demonstrates that FPP blocks lovastatin-induced increases in RhoB mRNA. Since GGPP synthesis requires both FPP and IPP, the finding that FPP alone can prevent lovastatin-induced upregulation of RhoB reveals FPP's regulatory activity. This finding is highly significant because of the central role for FPP in isoprenoid metabolism.

There is precedence in the literature for regulatory influence exerted by nonsterol isoprenoid species. Sterols are involved in the regulation of expression and activities of many proteins such as HMG-CoA reductase, FPP synthase, squalene synthase, and low-density lipoprotein receptor (*25*, *26*). For HMG-CoA reductase, this regulation is mainly transcriptional but also translational and post-translational (*26*−*28*). Nonsterol isoprenoid species have thus far been described to alter protein degradation only for HMG-CoA reductase. Farnesol, which is the alcohol derived from FPP via the enzyme farnesyl pyrophosphatase (*29*, *30*), has been identified as the isoprenoid species responsible for increasing the degradation of HMG-CoA reductase (*29*, *31*, *32*). Nonsterol isoprenoid species may also decrease HMG-CoA reductase mRNA translation but have no effect on transcription (*26*, *33*, *34*). In yeast, recent studies have suggested a more direct role for FPP, rather than farnesol, in regulating HMG-CoA reductase degradation; a mutant strain lacking the farnesyl pyrophosphatases LPP1 and DPP1 displayed responses to FPP similar to those of the wild type (*35*). Our studies more broadly show that mevalonate depletion induces upregulation of RhoA by increasing translation and decreasing protein degradation (*8*) but that RhoA mRNA levels are not altered (Figure 4). Importantly, our studies demonstrate that GGPP regulates RhoA protein levels post-transcriptionally (Figure 2). While there is the suggestion that FPP alone shares this property, the finding that FPP and IPP more completely mimic GGPP's effects indicates that GGPP is the primary isoprenoid for this regulation. As in the case of FPP, where the effects may be mediated by farnesol, the degree to which geranylgeraniol contributes to these regulatory processes remains to be determined.

Further evidence in support of our hypothesis that the lovastatin-induced upregulation of Ras and Ras-related proteins results from depletion of specific isoprenoids rather than from global inhibition of protein isoprenylation is shown in Figures 5−7. The selective or combined inhibition of FPTase with FTI-277 and GGPTase with GGTI-286 does not replicate the increase in Ras and RhoA protein synthesis (Figure 5), the decrease in Ras and RhoA degradation (Figure 5), the increase in RhoB mRNA (Figure 6), or the increase in total levels of Ras, Rap1a, RhoA, and RhoB (Figure 7) observed with mevalonate depletion. Concurrent treatment with both lovastatin and isoprenyl transferase inhibitors as well as FPP or GGPP allowed for dissection of the inhibition of isoprenylation from isoprenoid depletion. The upregulation of RhoA and RhoB in response to mevalonate depletion was mitigated by FPP and GGPP with ongoing inhibition of isoprenyl transferases (Figure 8). Furthermore, both FPP and GGPP prevented lovastatin-induced upregulation of RhoA and RhoB in the presence of squalene synthesis inhibition (Figure 3b). In aggregate, these results strongly support the role of isoprenoid pyrophosphates as potent regulators of the expression of Ras and Ras-related proteins.

The use of HMG-CoA reductase, FPP synthase, squalene synthase, FPTase, and GGPTase inhibitors has allowed us to expand the role for isoprenoids to influence metabolic processes. This manipulation and the observed upregulation of Ras and Ras-related proteins with the HMG-CoA reductase inhibition suggest that in uninhibited cells individual isoprenoids serve to negatively regulate expression of these proteins. While these experiments focus on Ras and Ras-related proteins, it seems likely that this regulation will be applicable to other isoprenylated proteins. This novel role for FPP and GGPP increases the complexity of the relationship between isoprenylated proteins and the isoprenoid biosynthetic pathway. That RhoB is regulated by both FPP and GGPP is significant due to the ability of RhoB to be either farnesylated or geranylgeranylated (*36*). Given the distinct functions of the farnesylated and geranylgeranylated forms of RhoB (*37*, *38*), it is possible that the relative availability of FPP and GGPP serves to regulate both the expression and the function of RhoB. The four proteins chosen for study are related, although RhoB is encoded by an immediate−early inducible gene (*39*) whereas the others are considered to be constitutively expressed. That these related proteins are differentially regulated by isoprenoid pyrophosphates may be indicative of underlying hierarchical relationships between these proteins. Further studies will reveal the consequences of this regulation on cellular processes.

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