Mol Neurobiol (2012) 46:28–40
DOI 10.1007/s12035-012-8249-x

Prenylation of Rho G-Proteins: a Novel Mechanism Regulating Gene Expression and Protein Stability in Human Trabecular Meshwork Cells

Evan B. Stubbs Jr. · Cynthia L. Von Zee

Received: 21 December 2011 / Accepted: 20 February 2012 / Published online: 7 March 2012

Abstract Endogenous prenylation with sesquiterpene or diterpene isoprenoids facilitates membrane localization and functional activation of small monomeric GTP-binding proteins. A direct effect of isoprenoids on regulation of gene expression and protein stability has also been proposed. In this study, we determined the role of sesquiterpene or diterpene isoprenoids on the regulation of Rho G-protein expression, activation, and stability in human trabecular meshwork (TM) cells. In both primary and transformed human TM cells, limiting endogenous isoprenoid synthesis with lovastatin, a potent HMG-CoA reductase inhibitor, elicited marked increases in RhoA and RhoB mRNA and protein content. The effect of lovastatin was dose-dependent with newly synthesized inactive protein accumulating in the cytosol. Supplementation with geranylgeranyl pyrophosphate (GGPP) prevented, while inhibition of geranylgeranyl transferase-I mimicked, the effects of lovastatin on RhoA and RhoB protein content. Similarly, lovastatin-dependent increases in RhoA and RhoB mRNA expression were mimicked by geranylgeranyl transferase-I inhibition. Interestingly, GGPP supplementation selectively promoted the degradation of newly synthesized Rho proteins which was mediated, in part, through the 20*S* proteasome. Functionally, GGPP supplementation prevented lovastatin-dependent decreases in actin stress fiber organization while selectively facilitating the subcellular redistribution of accumulated Rho proteins from the cytosol to the membrane and increasing RhoA activation. Post-translational prenylation with geranylgeranyl diterpenes selectively facilitates the expression, membrane translocation, functional activation, and turnover of newly synthesized Rho proteins. Geranylgeranyl prenylation represents a novel mechanism by which active Rho proteins are targeted to the 20*S* proteasome for degradation in human TM cells.

Keywords Trabecular meshwork · Rho · G-proteins · Isoprenoids

E. B. Stubbs Jr. (✉) · C. L. Von Zee
Research Service, Department of Veterans Affairs,
Edward Hines Jr. VA Hospital,
Hines, IL 60141, USA
e-mail: evan.stubbs@va.gov

E. B. Stubbs Jr. · C. L. Von Zee
Department of Ophthalmology, Stritch School of Medicine,
Loyola University Chicago,
Maywood, IL 60153, USA

Introduction

Isoprenoids and their derivatives are a family of naturally occurring terpenoids synthesized as key constituents of membranes, vitamins, pheromones and reproductive hormones, oxidative phosphorylation and photosynthesis, and components of signal transduction pathways affecting gene expression by organisms as diverse as bacteria, fungi, insects, plants, and mammals [1]. As comprehensively reviewed in this series by Wood et al., the primary source of bioactive sesquiterpene (farnesyl) and diterpene (geranylgeranyl) isoprenoids in mammalian cells is the cholesterol biosynthetic pathway. Farnesyl and geranylgeranyl isoprenoids function as post-translational modifiers of a variety of intracellular proteins including nuclear lamins A and B, rhodopsin kinase, γ-subunits of heterotrimeric GTP-binding proteins (G-proteins), as well as small monomeric Ras and Ras-related G-proteins [2]. Post-translational prenylation of small G-proteins proceeds by a multistep process involving transferase-catalyzed addition of farnesyl or

geranylgeranyl isoprenoids to conserved cysteine residues within a unique carboxy terminal-CaaX motif [2]. Largely considered an irreversible modification, cleavage of the resulting farnesylcysteine- or geranylgeranylcysteine-thioether bond may occur; however, in some prenylated proteins through the action of a prenylcysteine lyase, releasing free farnesal or geranylgeranial aldehydes [3], the significance of which remains unclear.

Although the biochemical consequences of post-translational prenylation remains to be fully elucidated, one biophysical attribute is to enhance small G-protein hydrophobicity thereby facilitating intracellular membrane localization and subsequent activation [4, 5]. Small G-proteins, including those belonging to the Rho family, regulate a diverse array of intracellular signaling pathways which affect vesicle transport/trafficking, endocytosis, cell cycle progression, cell contractility, and formation of stress fibers or focal adhesions [6, 7]. The Rho family of G-proteins consists of over 20 members, categorized into eight distinct subfamilies, of which Rho, Rac, and Cdc42 are the best studied. The Rho subfamily consists of RhoA, RhoB, and RhoC [8]. RhoA and RhoC are exclusively geranylgeranylated, whereas RhoB can accept either farnesyl or geranylgeranyl isoprenoids. The diversity in function and subcellular localization of Rho G-proteins is dictated by lipid modifications including post-translational isoprenylation [9] and in some cases (RhoB) palmitoylation [10, 11].

Within the human eye, normal intraocular pressure (~15 mmHg) is established and maintained through a balance between production and outflow of aqueous humor (AH). In healthy eyes, outflow of AH proceeds by a conventional pathway involving the trabecular meshwork (TM) at the iridocorneal angle. A percentage of AH also leaves the eye by an unconventional (uveoscleral) outflow route. The percent contribution of AH exiting the eye through the uveoscleral outflow pathway declines with advancing age. Indirect measurements of uveoscleral outflow in adolescent (24–30 years old) humans ranges between 0.8 and 1.52 μl/min, representing 36–54 % of the total AH outflow [12]. By comparison, outflow of AH by the uveoscleral pathway in elderly adult humans represents <10 % of the total AH outflow, with the balance of AH exiting the eye through the conventional TM pathway.

Within TM cells, one essential function of membrane-anchored RhoA G-proteins is to promote actin stress fiber organization [13–15]. Aberrant activation of RhoA enhances actin-mediated contractile tone of TM cells resulting in reduced AH outflow and subsequent elevation of intraocular pressure [16]. Inhibition of Rho G-proteins, or their downstream Rho effectors such as Rho kinase, has been shown to improve AH outflow and reduce intraocular pressure [17–22]. Consequently, selective inhibitors of Rho signaling are aggressively being explored as potential therapeutic agents for the management of elevated intraocular pressure associated with some forms of glaucoma [23, 24].

An alternative role for isoprenoids within mammalian cells may involve regulation of gene expression or protein stability [25–27]. We recently reported that statins elicit a marked accumulation of Rho proteins in the cytosol of human TM cells, in part, by enhancing expression of Rho G-protein isoforms [28, 29]. Statins, widely used for the treatment of hypercholesterolemia [30], are now recognized as indirect inhibitors of Rho signaling [31]. By inhibiting HMG-CoA reductase, statins block endogenous synthesis of farnesyl- and geranylgeranyl isoprenoids and thereby disrupt post-translational prenylation of proteins, including Rho [31–33]. As a consequence, Rho G-proteins remain compartmentalized to the cytosol in an inactive (GDP-bound) state. Some immature forms of Rho, however, may maintain partial function [34, 35]. These and other studies have generated considerable interest in evaluating the role of isoprenoids as potential therapeutic targets for the management of a wide variety of neurological diseases, including Alzheimer's disease [36–41].

In this study, we discuss the functional consequence of post-translational prenylation in regulating Rho G-protein expression, activation, and stability in human TM cells. Post-translational geranylgeranylation is found to selectively alter Rho mRNA expression while targeting Rho proteins for proteasomal degradation, representing a novel function of post-translational protein prenylation in human TM cells. We introduce the concept of prenylation itself as a regulator of Rho G-protein gene expression and turnover in human TM cells.

Materials and Methods

Human TM Cell Culture

The use of human cadaver material in this study was approved by the Edward Hines Jr. VA and Loyola University Chicago institutional review boards. Fresh cadaver corneoscleral rims from normal subjects were obtained (Illinois Eye Bank, Chicago, IL) at time of corneal transplant, and primary human TM cells were prepared using a collagenase-free procedure as previously described [28, 42]. The purity of primary TM cell cultures typically exceeded 95 % as routinely determined by cell morphology. Human TM cells from a male glaucomatous patient (GTM3) that were SV40-transformed were a generous gift from Alcon Laboratories (Ft. Worth, TX). Human primary TM cell cultures were established on Falcon Primaria flasks (BD Biosciences, San Jose, CA) in Eagle's minimum essential medium containing 2 mM L-glutamine supplemented with 5 % adult bovine serum, 10 % fetal bovine serum, 0.1 % gentamycin,

1 % amphotericin B, and a mixture of essential (Life Technologies, Grand Island, NY) and nonessential amino acids. Transformed human GTM3 cells were cultured in Dulbecco's modified Eagle's medium containing 4 mM GlutaMAX-I supplemented with 10 % fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary and transformed cultures were maintained at 37°C under an atmosphere of 5 % CO_2/95 % air. Unless indicated otherwise, all chemicals/inhibitors were obtained from Sigma-Aldrich Chemical Co, St. Louis, MO.

Treatment of TM Cell Cultures

Prior to use, lovastatin (EMD Chemicals, Gibbstown, NJ) was chemically activated by alkaline hydrolysis as described previously [28, 29]. Stock solutions of activated lovastatin hydroxy acid (4 mg/ml) were stored at −20°C until use. Confluent cultures of human TM cells were treated for 24 h with vehicle (0.01 % ethanol) or activated lovastatin (10 μM) for 24 h unless noted otherwise to enhance endogenous accumulation of Rho proteins [29]. For transcription or translation experiments, cell cultures were pretreated (pulsed for 1 h, 37°C) with actinomycin D (0.5 μg/ml) or cycloheximide (5 μM), respectively. To inhibit post-translational prenylation of Rho G-proteins, human TM cell cultures were incubated in the presence of selective cell-permeable farnesyl transferase (FTI-277, 10 μM) or geranylgeranyl transferase-I (GGTI-298, 10 μM) inhibitors. Rho protein translocation studies were conducted using lovastatin pretreated cells that were cultured for an additional 0–6 h in fresh media supplemented with farnesyl pyrophosphate (FPP, 10 μM), or geranylgeranyl pyrophosphate (GGPP, 10 μM). To evaluate the effect of exogenous isoprenoids on Rho protein stability, lovastatin pretreated cells were cultured for an additional 0–24 h in fresh media containing cycloheximide (5 μM) supplemented without or with GGPP (10 μM). The mechanism facilitating Rho protein degradation was assessed using lovastatin pretreated cells cultured for an additional 24 h in fresh cycloheximide-containing media supplemented with select inhibitors of lysosomal, autophagic, or proteasomal degradation pathways. In all cases, TM cell viability was routinely determined by trypan blue dye exclusion and was consistently >90 %.

Subcellular Fractionation

Treated cells were harvested (0.05 % trypsin–EDTA), washed once in fresh media, and lysates prepared by re-suspending washed cell pellets in deionized water supplemented with a commercial cocktail of protease inhibitors (Roche Applied Science, Indianapolis, IN). Freshly prepared lysates were subjected to either subcellular fractionation or aliquots were stored at −80°C until use. Particulate (crude membranes) and soluble (cytosolic) subcellular fractions were prepared from GTM3 cell lysates as previously described [28, 29]. Protein concentrations in cell lysates and prepared subcellular fractions were determined by the BCA method (Thermo Scientific, Rockford, IL) using bovine serum albumin as the standard.

Real-Time RT-PCR

Total RNA was extracted from human TM cells using TRIzol reagent, and 5 μg was reverse-transcribed using Super Script III First Strand Synthesis system (Life Technologies). RhoA-, RhoB-, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific cDNA sequences were amplified by real-time (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA) quantitative RT-PCR using a Mini-Opticon PCR detection system with published human-specific primer pairs as previously described [28]. Human-specific GAPDH primers were used as a reference control. For each sample, the specificity of the real-time reaction product was determined by melting curve analysis. Reaction efficiencies were typically >90 %. The endogenous expression of GAPDH was unaltered by drug treatment. Relative fold changes in gene expression in each sample were therefore normalized to expressed levels of GAPDH.

Immunoblot Analysis

Proteins (20 μg protein per lane) in cell lysates or subcellular fractions were resolved by SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes as previously described [28]. Membranes were blocked and incubated overnight at 4°C in the presence of a 1:200 dilution of mouse anti-RhoA, rabbit anti-RhoB, mouse anti pan-Ras primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or 1:1000 dilution of rabbit anti-Rac1/2/3 or rabbit anti-Cdc42 primary antibody (Cell Signaling Technology, Danvers, MA). Washed membranes were incubated for 1 h at 23°C in the presence of appropriate horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,500 dilution) or goat anti-rabbit IgG (1:10,000 dilution) secondary antibody (Jackson ImmunoResearch Labs, Inc., West Grove, PA). In some cases, immunostained blots were stripped with Restore Western Blot stripping buffer (Thermo Scientific) between applications to quantify relative changes in Rho protein isoform content on the same membrane. To confirm equal protein loading, all immunoblots were separately incubated overnight at 4°C in the presence of a 1:10,000 dilution of rabbit anti-GAPDH primary antibody (Trevigen, Gaithersburg, MD).

Membranes were subsequently washed and incubated for 1 h at 23°C in the presence of horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) secondary antibody. In all cases, immunostained proteins were visualized by enhanced chemiluminescence (Thermo Scientific). Relative changes in RhoA or RhoB protein content were quantified by densitometry and normalized to total GAPDH content.

RhoA Activation Assay

The content of active (GTP-bound) RhoA protein in GTM3 cell lysates was determined by ELISA using a commercially available RhoA-specific G-LISA™ activation assay kit (Cytoskeleton Inc., Denver, CO). Briefly, cell lysates (1.4 mg/ml) were incubated for 30 min at 4°C in microtiter wells precoated with a RBD domain of Rho family effector proteins. Washed wells were incubated for 45 min at 23°C in the presence of a 1:250 dilution of mouse anti-RhoA antibody followed by incubation with a 1:62.5 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody. The content of GTP-bound RhoA present in cell lysates was quantified spectrophotometrically at 490 nm.

Filamentous Actin Organization

GTM3 cells were cultured on Nunc Lab-Tek II (Thermo Fisher Scientific, Rochester, NY)-chambered cover slips and pretreated for 24 h without (0.01 % ethanol) or with activated lovastatin (10 μM). Lovastatin pretreated cells were washed and subsequently incubated for additional 6 h in the presence of vehicle (0.3 % methanol) or GGPP (10 μM). Treated cells were fixed for 15 min at 23°C by immersion in phosphate-buffered (pH 7.4) 4 % paraformaldehyde. Filamentous actin stress fiber organization was determined by immunofluorescence using AlexaFluor 488-conjugated phalloidin as previously described [29]. All confocal images were captured using identical settings and fluorescence intensity of phalloidin-positive cells was semiquantified using ImageJ (v1.43u), expressed as background-corrected integrated fluorescent density.

Statistical Analysis

Unless otherwise specified, results are expressed as mean ± SD of triplicate cultures, repeated at least one additional time. Statistical significance was determined using one-way ANOVA followed by a Bonferroni's or Dunnett's multiple comparison post-test analysis. In all cases, $p<0.05$ was considered statistically significant.

Results

Lovastatin Increases Rho G-Protein Content

Human primary or transformed TM cells cultured in the presence of activated lovastatin expressed marked increases in the content of Ras homolog (Rho) proteins (Fig. 1). Lovastatin-dependent changes in G-protein expression were not unique to the Rho subfamily of proteins, as lovastatin similarly elicited increases in the content of Cdc42, Rac1/2/3, as well as Ras G-proteins (Fig. 1b). RhoA was found to be constitutively expressed in resting vehicle-treated transformed cells, while increasing robustly in response to lovastatin treatment (Fig. 1c, d). By comparison, primary and transformed TM cells at rest did not express measurable levels of total RhoB G-proteins. Similar to RhoA, however, lovastatin treatment elicited a marked increase in RhoB content (Fig. 1). Cotreatment of TM cell cultures with mevalonate, the immediate metabolic product of HMG-CoA reductase, completely prevented lovastatin-dependent increases in RhoA and RhoB (Fig. 1c). Mevalonate treatment alone had no effect on RhoA constitutive expression, minimizing a direct effect of this metabolite on Rho protein expression.

In resting transformed TM cells, Rho was found to be predominantly associated with cell membranes (Fig. 2). In contrast, lovastatin elicited a decrease in membrane-associated Rho proteins with a disproportionate accumulation of both RhoA and RhoB in the cell cytosol (Fig. 2a). The effect of lovastatin was dose-dependent, with measurable increases in soluble RhoA observed starting at ~100 nM. Interestingly, higher concentrations of lovastatin (~1 μM) were needed to alter soluble RhoB content in these cells. Membrane-associated RhoB G-proteins were not detectable in vehicle-treated cells.

By altering the subcellular localization of Rho, lovastatin may influence the amount of functionally active (GTP-bound) Rho G-proteins present in TM cells. Measurable quantities of GTP-bound RhoA were present in vehicle-treated transformed TM cells (Fig. 2b). At 24 h, when lovastatin is seen to increase the content of total soluble RhoA, functionally active (GTP-bound) RhoA was, however, significantly ($p<0.001$) *reduced* compared to vehicle-treated controls (Fig. 2b). These data emphasize that statins elicit marked increases in largely *inactive* (GDP-bound) cytosolic Rho proteins.

Geranylgeranyl Pyrophosphates Selectively Prevent Lovastatin Increases in Rho G-Protein Content

Supplementing primary or transformed human TM cell cultures with the diterpene GGPP prevented lovastatin-dependent increases in RhoA and RhoB content (Fig. 3a,

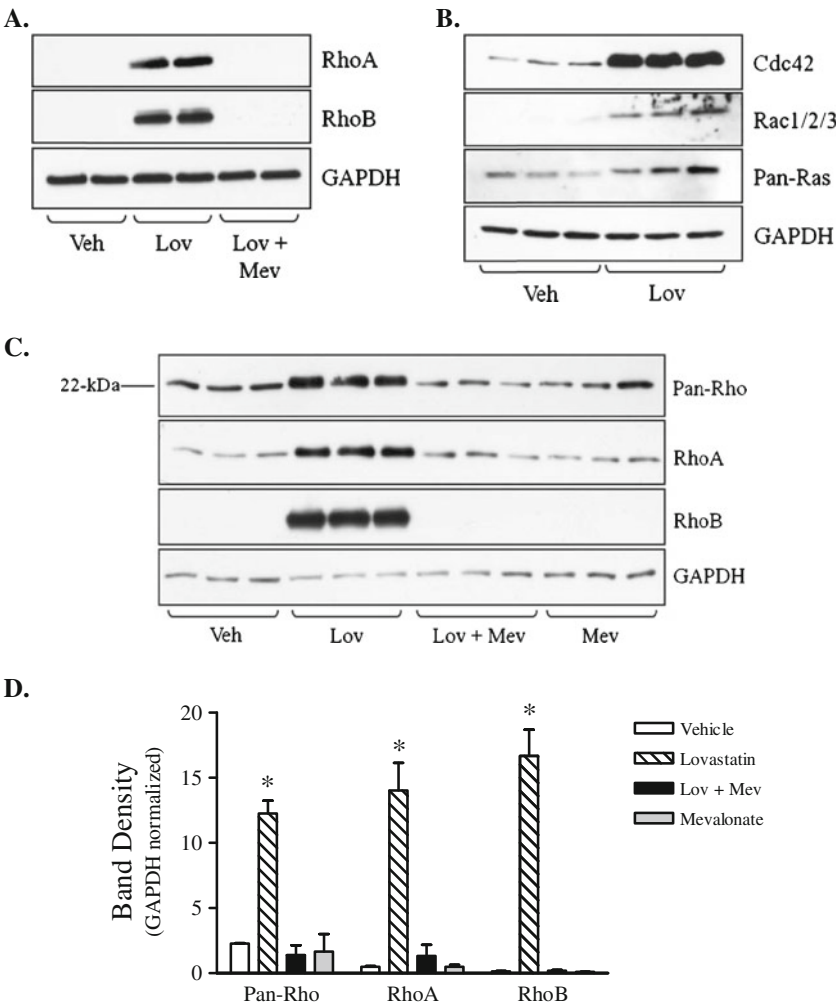

Fig. 1 Lovastatin increases the content of Ras homolog G-proteins. Immunoblots of G-proteins expressed in cell lysates from **a** primary or **b**, **c** transformed human TM cells cultured in the absence (*Veh*, 0.01 % ethanol) or presence of lovastatin (*Lov*, 10 μM) for 24 h (transformed) or 48 h (primary) without or with mevalonate (*Mev*, 5 mM) as indicated. GAPDH content is shown for comparison as a loading control. Data are representative of 1–3 separate experiments. **d** Quantitative densitometric comparison of the results shown in **c** normalized to GAPDH and expressed as the mean ± SD (n=3). Vehicle (*open bar*), lovastatin (*hatched bar*), lovastatin + mevalonate (*solid bar*), mevalonate (*gray bar*). *p<0.01; one-way ANOVA with Dunnett's post hoc analysis

b). This effect was selective for GGPP, as supplementing cultures with the sesquiterpene FPP had no effect on lovastatin-dependent changes in Rho G-protein expression (Fig. 3d). Experimentally chasing lovastatin-treated cultures with GGPP facilitated, in a time-dependent manner, the translocation of accumulated cytosolic RhoA and RhoB G-proteins to the membrane (Fig. 3c). Chasing cultures with FPP did not, however, facilitate translocation of cytosolic Rho G-proteins to the membrane [29].

The mechanism by which geranylgeranyl isoprenoid supplementation affects lovastatin-dependent Rho G-protein expression was determined by treating transformed TM cell cultures with cell-permeable inhibitors specific for farnesyl transferase (FTI-277) or geranylgeranyl transferase-I (GGTI-298). Primary or transformed TM cells treated with GGTI-298 exhibited a significant increase in RhoA and RhoB content, mimicking the effect of lovastatin (Fig. 4a, b). In contrast, the content of RhoA and RhoB in FTI-277-treated transformed TM cells was similar to vehicle-treated controls (Fig. 4b, c).

Lovastatin Increases Rho G-Protein mRNA Content

Primary human TM cells treated with lovastatin exhibited increases in the relative content of both RhoA and RhoB isoform-specific mRNA [28]. Transformed human TM cells responded similarly to lovastatin (Fig. 5a, b), which was preventable by coincubation with mevalonate [28]. In transformed cells, the stability of newly expressed RhoA mRNA ($t_{1/2}$=>24 h) was substantially greater than that of RhoB mRNA ($t_{1/2}$=<2 h). Consistent with previous reports [43], lovastatin had no effect on the half-life of newly expressed RhoA or RhoB mRNA (n=2; data not shown). However, supplementing transformed cultures with GGPP, but not FPP, prevented lovastatin increases in RhoB mRNA content (Fig. 5b). The effect of exogenously added GGPP on lovastatin-dependent changes in RhoA mRNA content was not significant. Interestingly, transformed TM cells treated with GGTI-298 mimicked the effect of lovastatin on both RhoA and RhoB mRNA expression (Fig. 5c, d). However, treating transformed cells with FTI-277 had no effect on RhoA or RhoB mRNA expression (Fig. 5d).

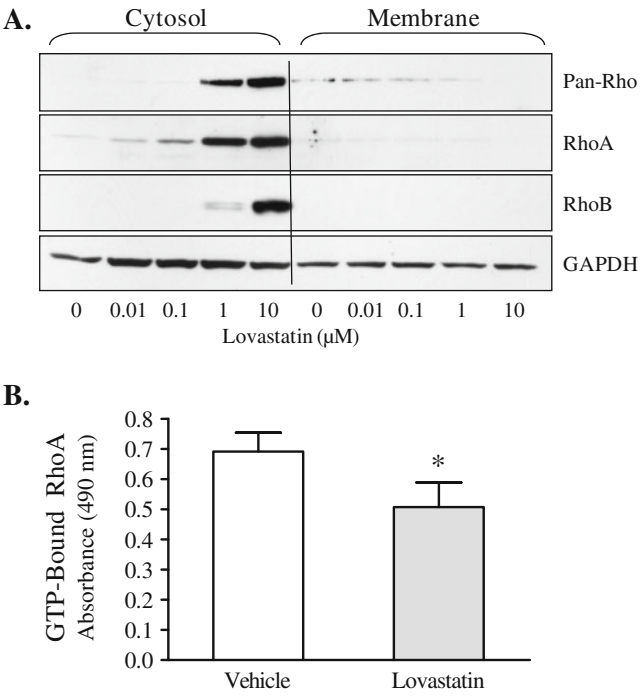

Fig. 2 Dose-dependent localization of Rho proteins to the cell cytosol by lovastatin. **a** Immunoblot of pan-Rho, RhoA, and RhoB protein expression and subcellular distribution in transformed TM cells incubated for 24 h in the absence (0 μM) or presence of lovastatin (0.01–10 μM) as indicated. Soluble (*cytosol*) and particulate (*crude membrane*) fractions were prepared as described in "Materials and Methods". Data shown are representative of three separate experiments. **b** Transformed TM cells were incubated with vehicle (0.01 % ethanol) or lovastatin (10 μM) for 24 h as indicated and the content of GTP-bound RhoA in cell lysates was determined by G-LISA. Data shown are expressed as the mean±SD from a single experiment performed in triplicate. *$p<0.0001$; unpaired Student's *t* test

Unexpectedly, pretreating cultures with actinomycin D, an inhibitor of gene transcription, had a small but statistically insignificant effect on lovastatin-dependent increases in RhoA G-protein content (Fig. 6a, c). In marked contrast, actinomycin D pretreatment completely prevented lovastatin-dependent increases in RhoB G-protein expression (Fig. 6a, c). Inhibiting de novo protein synthesis with cycloheximide, by comparison, blocked lovastatin-dependent increases in both RhoA and RhoB G-protein expression (Fig. 6b, d).

Geranylgeranylation Selectively Targets Rho G-Proteins for Proteasomal Degradation

In addition to facilitating the subcellular redistribution of Rho G-proteins, we examined the possibility that geranylgeranyl isoprenoids may alter Rho G-protein stability in human TM cells. Transformed TM cells pretreated with lovastatin and subsequently chased in the presence of cycloheximide exhibited a steady decline in Rho G-proteins

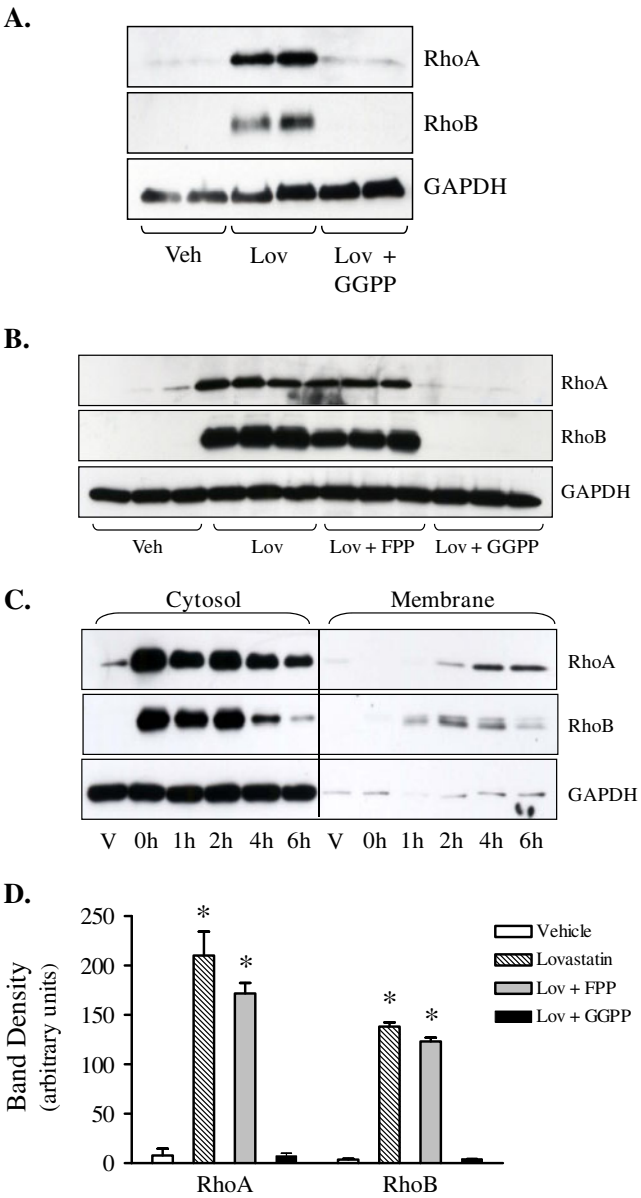

Fig. 3 Geranylgeranyl pyrophosphate prevents lovastatin-mediated increases in RhoA and RhoB protein. Immunoblots of RhoA and RhoB proteins expressed in cell lysates from **a** primary or **b** transformed human TM cells incubated in the absence (*Veh*, 0.01 % ethanol) or presence of lovastatin (*Lov*, 10 μM) without or with farnesyl pyrophosphate (*Lov+FPP*, 10 μM) or geranylgeranyl pyrophosphate (*Lov + GGPP*, 10 μM) supplementation for 24 h (transformed) or 48 h (primary) as indicated. **c** Lovastatin pretreated (24 h) transformed TM cells were chased for an additional 0–6 h, as indicated, with GGPP (10 μM). GAPDH content is shown for comparison as a loading control. Immunoblots shown are representative of one to two separate experiments. **d** Quantitative densitometric comparison of the results shown in **b** expressed as the mean ± SD ($n=3$). Vehicle (*open bar*), lovastatin (*hatched bar*), lovastatin + FPP (*gray bar*), lovastatin + GGPP (*solid bar*). *$p<0.01$; one-way ANOVA with Dunnett's post hoc analysis

(Fig. 7). Using a best-fit exponential decay analysis, the apparent half-life ($t_{1/2}$) of RhoA G-proteins in TM cells is

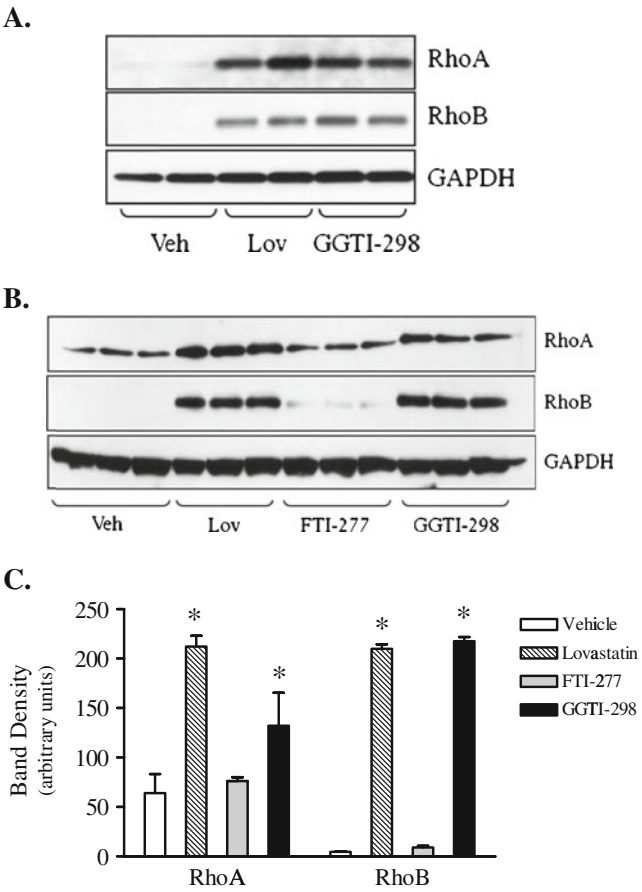

Fig. 4 Inhibition of geranylgeranyl transferase-I mimics lovastatin-dependent increases in RhoA and RhoB protein. Immunoblots of RhoA and RhoB proteins expressed in cell lysates from **a** primary or **b** transformed human TM cells incubated in the absence (*Veh*, 0.01 % ethanol) or presence of lovastatin (*Lov*,10 μM), farnesyl transferase inhibitor (*FTI-277*, 10 μM), or geranylgeranyl transferase inhibitor-I (*GGTI-298*, 10 μM) for 24 h (transformed) or 48 h (primary) as indicated. Immunoblots shown are representative of one to two separate experiments each. GAPDH content is shown for comparison as a loading control. **c** Quantitative densitometric comparison of the results shown in **b** expressed as the mean ± SD (n=3). Vehicle (*open bar*), lovastatin (*hatched bar*), FTI-277 (*gray bar*), GGTI-298 (*solid bar*). *p<0.01; one-way ANOVA with Dunnett's post hoc analysis

approximately 13.2 h. By comparison, RhoB G-proteins are less stable, exhibiting an apparent half-life of 6.7 h. Leaving lovastatin in the media (Fig. 7, control) extended the half-life of both RhoA ($t_{1/2}$=19.4 h) and RhoB ($t_{1/2}$=11.2 h). Addition of GGPP (but not FPP) to the lovastatin containing chase media, however, selectively facilitated degradation (RhoA $t_{1/2}$=3.9 h; RhoB $t_{1/2}$=6.7 h) of RhoA and RhoB G-proteins (Fig. 7).

The mechanism by which GGPP facilitates Rho G-protein degradation in human TM cells was assessed with a combination of lysosome, autophagosome, or ubiquitin-proteasome system inhibitors. The presence of lysosomal inhibitors NH_4Cl (0–30 μM) or chloroquine (0–10 μM) had no effect on GGPP-facilitated Rho G-protein degradation (data not shown). Similarly, the addition of autophagosome inhibitors 3-methyladenine (0–10 mM) or wortmannin (0–10 μM) did not alter GGPP-facilitated Rho G-protein degradation (data not shown). In contrast, the 20*S* proteasome inhibitor epoxomicin (10 μM) protected Rho G-proteins from GGPP-facilitated degradation (Fig. 8).

Geranylgeranyl Isoprenoids Functionally Restore Stress Fiber Organization

Transformed TM cells cultured for 24 h in the presence of lovastatin and subsequently chased with vehicle (0.3% methanol) exhibited a restoration of GTP-bound RhoA that was significantly enhanced, compared with vehicle controls, by GGPP (Fig. 9a). These findings are consistent with GGPP-facilitated redistribution to cell membranes (Fig. 3). To determine whether Rho G-protein reactivation in TM cells elicits functional consequences, we semiquantified restoration of filamentous actin stress fiber organization after removal of lovastatin. Resting TM cells express numerous phalloidin-positive filamentous actin stress fibers (Fig. 9b). In contrast, cells pretreated with lovastatin exhibited a marked reduction in filamentous actin stress fiber staining along with qualitative changes in cell morphology (Fig. 9b). Removal of lovastatin produced a modest increase of phalloidin-positive filamentous actin stress fiber staining that was significantly enhanced by GGPP (Fig. 9b).

Discussion

In this study, the role of sesquiterpene (C_{15} farnesyl) and diterpene (C_{20} geranylgeranyl) isoprenoids on the regulation of RhoA and RhoB G-protein expression, activation, and protein stability was determined in human primary and transformed TM cells. Limiting endogenous isoprenoid availability by inhibiting HMG-CoA reductase with lovastatin elicited marked increases in RhoA and RhoB mRNA and protein expression. Exogenously added geranylgeranyl isoprenoids selectively prevented, while inhibitors of geranylgeranyl prenylation mimicked, lovastatin-dependent increases in Rho G-protein expression. Geranylgeranyl prenylation facilitated membrane translocation and functional reactivation of soluble inactive Rho G-proteins while concomitantly restoring actin stress fiber organization. Geranylgeranyl prenylation similarly facilitated the degradation of RhoA and RhoB proteins. Epoxomicin, a potent inhibitor of the 20*S* proteasome, prevented geranylgeranyl prenylation-enhanced degradation of Rho proteins. We propose that post-translational prenylation by diterpene geranylgeranyl isoprenoids represents a novel mechanism affecting the life cycle of Rho subfamily of G-proteins.

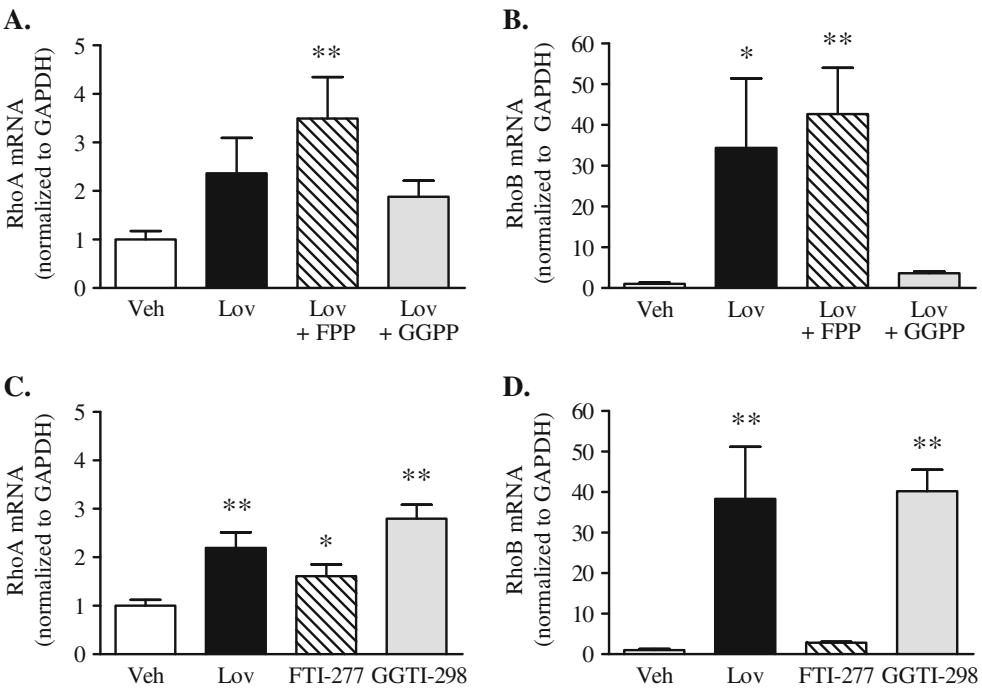

Fig. 5 Lovastatin-dependent increases in RhoA and RhoB mRNA content is mimicked by geranylgeranyl transferase-I. Relative fold changes in **a**, **c** RhoA and **b**, **d** RhoB mRNA content in transformed TM cells incubated (24 h) in the absence (*Veh*, 0.01 % ethanol) or presence of lovastatin (*Lov*, 10 μM), lovastatin plus farnesyl pyrophosphate (*Lov + FPP*, 10 μM), lovastatin plus geranylgeranyl pyrophosphate (*Lov + GGPP*, 10 μM), farnesyl transferase inhibitor (*FTI-277*, 10 μM), or geranylgeranyl transferase inhibitor (*GGTI-298*, 10 μM) as indicated. Data shown are GAPDH-normalized changes from a single experiment performed in triplicate and expressed as mean ± SD. *$p<0.05$; **$p<0.01$; one-way ANOVA with Dunnett's post hoc analysis

Beyond their role as intermediates in cholesterol biosynthesis, the 15-carbon farnesyl and 20-carbon geranylgeranyl isoprenoids serve to function as post-translational biophysical modifiers of small monomeric Ras and Ras homolog (Rho) G-proteins. Post-translational prenylation of small G-proteins facilitates intracellular membrane localization and subsequent activation required for proper cell signaling [4, 5]. A physiologic role of isoprenoids beyond enhancing membrane hydrophobicity has begun to emerge, including their participation as key regulators of HMG-CoA reductase

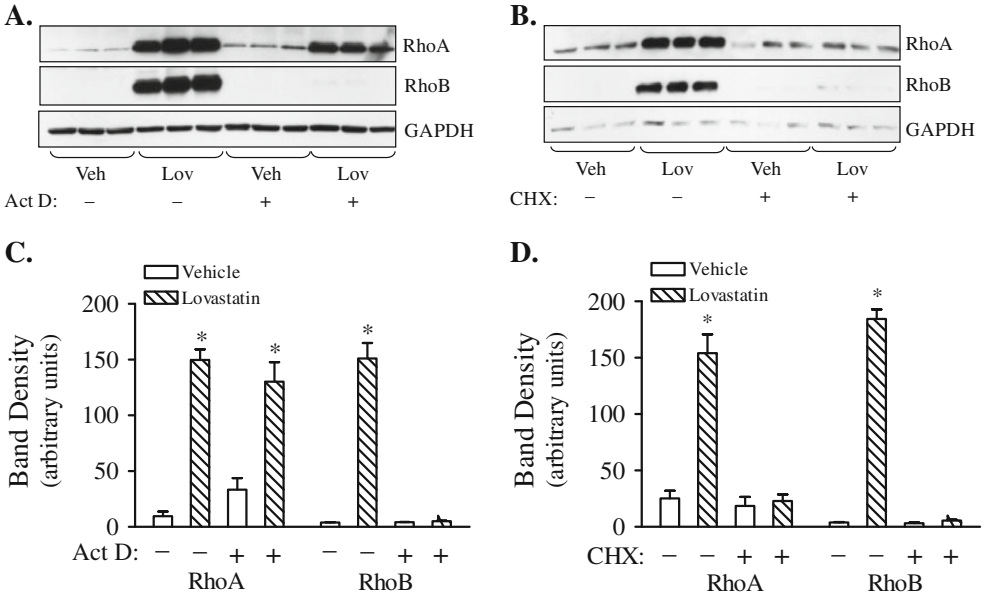

Fig. 6 Effect of transcriptional and translational inhibitors on lovastatin-mediated increases in RhoA and RhoB protein. Immunoblots of RhoA and RhoB proteins expressed in cell lysates from transformed TM cells pretreated (1 h) without (−) or with (+) **a** actinomycin D (*Act D*, 0.5 μg/ml) or **b** cycloheximide (*CHX*, 5 μM) and incubated for an additional 24 h in the absence (*Veh*, 0.01 % ethanol) or presence of lovastatin (*Lov*, 10 μM) as indicated. Immunoblots shown are from a single experiment performed in triplicate. GAPDH content is shown for comparison as a loading control. **c**, **d** Quantitative densitometric comparison of the results shown in **a**, **b** expressed as the mean ± SD. Vehicle (*open bar*), lovastatin (*hatched bar*). *$p<0.01$; one-way ANOVA with Dunnett's post hoc analysis

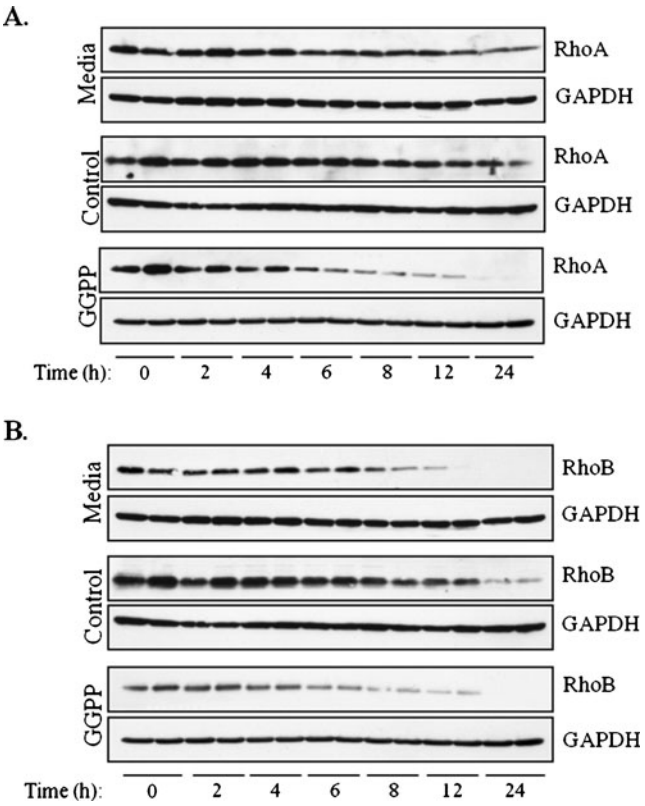

Fig. 7 Geranylgeranyl pyrophosphates accelerate degradation of RhoA and RhoB proteins. Immunoblots of **a** RhoA and **b** RhoB proteins expressed in cell lysates from transformed TM cells pretreated for 24 h with lovastatin (10 μM, 0 h) and chased for an additional 0–24 h in fresh media containing cycloheximide (5 μM) without (*Media*) or with lovastatin (10 μM) in the absence (*Control*) or presence of 10 μM GGPP (*GGPP*), as indicated. Data shown are from a single experiment performed in duplicate. Levels of GAPDH are shown for comparison as loading controls

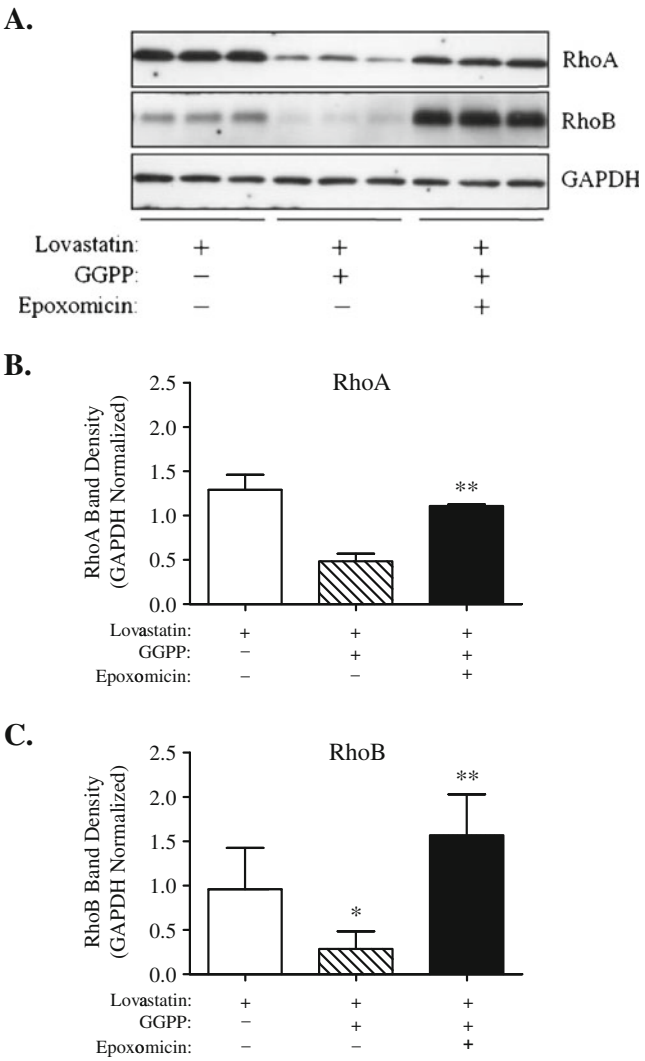

Fig. 8 Epoxomicin protects against geranylgeranyl pyrophosphate-accelerated degradation of RhoA and RhoB proteins. **a** Immunoblot of RhoA and RhoB proteins expressed in cell lysates from transformed TM cells pretreated with lovastatin (10 μM, 24 h) and chased for an additional 24 h in cycloheximide (5 μM)-supplemented media without or with GGPP (10 μM) or epoxomicin (10 μM), as indicated. Data shown are representative two separate experiments each performed in triplicate. **b**, **c** Quantitative densitometric comparison of the results shown in **a** expressed as the mean ± SD. Results normalized to GAPDH and expressed as mean ± SD. *$p<0.05$; **$p<0.01$; one-way ANOVA with a Dunnett's multiple comparison post hoc analysis

[25, 44] or monomeric G-protein [26, 27, 45] expression and turnover. Findings reported in this study strongly support a selective role for geranylgeranyl prenylation, but not farnesylation, in targeting RhoA and RhoB proteins to the 20*S* proteasome for degradation in human TM cells. Some of our findings, however, were obtained using SV40-transformed human TM cells originally harvested from a patient with glaucoma, raising the concern that the disease itself (or the process of cell transformation) may have altered normal TM cell metabolic responses. To address this possibility, key experiments were repeated using primary human TM cells harvested from otherwise healthy donors. We find qualitatively similar results in the replicated key experiments thereby minimizing this concern. This is not unexpected, since the transformed TM cell line used here has been previously well characterized and shown to express a phenotype consistent with differentiated non-transformed human primary TM cells [46]. We therefore propose that lovastatin-dependent changes in Rho expression seen here are not unique to cell transformation or cell origin but rather represent a general biochemical response to agents that limit de novo isoprenoid synthesis.

Through their ability to deplete cells of key isoprenoid species, statins have been shown to upregulate expression of Ras and several Ras-related G-proteins including Rap1a, Rab5, Rab7, RhoA, and RhoB [1]. The mechanism by which isoprenoids regulate Rho G-protein expression remains to be fully elucidated. Depleting key bioactive isoprenoid intermediates with statins may indirectly affect Rho G-protein expression by disrupting protein prenylation.

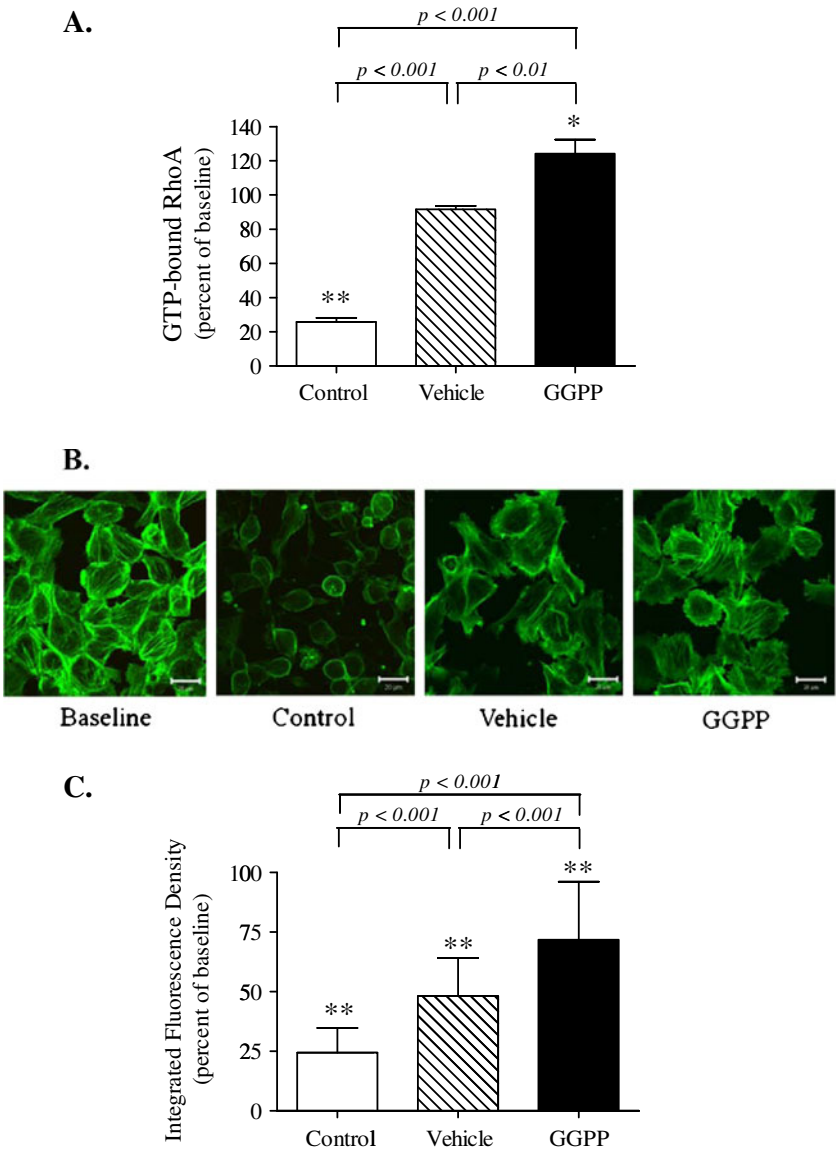

Fig. 9 Geranylgeranyl pyrophosphates facilitate functional reactivation of RhoA. **a** G-LISA of GTP-bound RhoA in transformed TM cells incubated with lovastatin (10 μM, 24 h, *Control*) or with lovastatin (10 μM, 24 h pretreatment) followed by 6 h post-treatment in the absence (Vehicle, 0.3% methanol) or presence of geranylgeranyl pyrophosphate (*GGPP*, 10 μM) as indicated. Data shown are the means ± SD of absorbance values expressed as percent baseline (no treatment controls) from a single experiment performed in triplicate. **b** Confocal images of phalloidin-positive actin stress fibers in transformed TM cells treated as described in **a**. **c** Images in **b** were semiquantified using ImageJ (version 1.43u). Data shown are the means ± SEM of background-corrected integrated fluorescent densities expressed as percent baseline (no treatment controls) from a single experiment (n=27–37 cells). *Bar*, 20 μm. *p<0.01; **p<0.001, one-way ANOVA with a Bonferroni multiple comparison post hoc analysis

Alternatively, FPP and GGPP isoprenoids and their derivatives may directly repress Rho gene expression [26]. In agreement with Holstein et al., we found that restoring isoprenoid levels with exogenous GGPP completely prevented statin-dependent increases in Rho G-protein accumulation in TM cells. However, selectively inhibiting geranylgeranyl transferase-I mimicked the effect of lovastatin on RhoA and RhoB gene expression in transformed TM cells. These findings differ from that of Holstein and Hohl, and strongly argue for a novel role of geranylgeranyl prenylation in the autoregulation of Rho G-protein expression in human TM cells.

Mechanisms regulating Rho G-protein turnover include ubiquitin-mediated proteasomal degradation. Cyclic nucleotide-dependent phosphorylation of serine 188 on RhoA is reported to enhance the stability of this protein through RhoGDI-mediated cytoplasmic compartmentalization in vascular smooth muscle cells [47]. Interestingly, sequestering active RhoA in the cell cytosol was found to protect against ubiquitin-mediated proteasomal degradation. In TM cells, as well as most mammalian cells, the ubiquitin–proteasome oligomeric complex plays an important role in regulating protein turnover [48, 49]. Targeting of RhoA for proteasomal degradation has been clarified, in other cell systems, and proceeds by Smurf1-catalyzed ubiquitination [50]. Recently, Chen et al. showed that RhoA can also be ubiquitinated by Cul3, a Cullin family scaffolding protein [51]. Knockdown of Cul3 results in abnormal actin stress fiber organization and altered cell morphology [51].

Whereas ubiquitination targets proteins for degradation by the 26*S* proteasome, other cytoplasmic proteasomal assemblies (20*S*) have been shown to selectively degrade oxidized proteins [52]. It is interesting to note that degradation of oxidized proteins by the 20*S* proteasome initially

proceeds by recognition of exposed hydrophobic amino acid surface residues. A functional consequence of post-translational prenylation is enhanced hydrophobicity of an otherwise soluble protein. We find that geranylgeranyl, but not farnesyl, isoprenoids selectively target Rho proteins to an epoxomicin-sensitive 20*S* proteasome in TM cells. Due to the selectivity of this effect, however, we speculate that this mechanism involves specific recognition of this diterpene rather than a general change in the proteins biophysical property. This is most evident when considering geranylgeranyl isoprenoid-facilitated turnover of RhoB, which itself is capable of accepting either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid [32].

Additional proteolytic systems which may participate in regulating Rho G-protein turnover in TM cells include calcium-regulated cysteine proteases (calpains), ATP-dependent mitochondrial proteases (e.g., Lon protease), and autophagy by lysosomal compartmentation [48]. However, specific inhibitors of the autophagosomal/lysosomal proteolytic pathways used here failed to protect Rho proteins from geranylgeranyl-facilitated degradation. Collectively, our findings suggest that geranylgeranyl prenylation preferentially targets RhoA and RhoB G-proteins in TM cells to the 20*S* proteasome, but not to the autophagosome or the lysosome. Proteasomal targeting of RhoB may itself be a unique characteristic of TM cells, since a recent study supports degradation of prenylated, palmitoylated RhoB through an endo-lysosomal pathway [53].

The functional significance of the present findings are underscored by the dominant role that RhoA, and possibly RhoB, plays in regulating filamentous actin stress fiber organization and cellular contractility of human TM cells [54]. Marked qualitative changes in TM cell shape and reduced F-actin cytoskeletal organization are observed following lovastatin treatment [28, 29]. The disruptive effect of lovastatin on cell shape and F-actin organization was prevented by exogenous geranylgeranyl pyrophosphate, inferring post-translational prenylation of Rho G-proteins.

Inhibition of Rho G-proteins, or their downstream Rho effectors such as Rho-associated coiled coil-forming protein kinases (ROCK), has been shown to improve AH outflow and reduce intraocular pressure [17–22, 55, 56]. Moreover, inhibition of Rho signaling with C3 exoenzyme or expression of dominant-negative RhoA enhanced outflow in human anterior segment cultures [18, 57]. Whereas the optimal molecular target remains to be identified, these studies collectively support proof of concept. In this regard, a retrospective matched case–control study using male patients >50 years of age with newly diagnosed glaucoma shows that chronic (>24 months) statin use to be associated with a lower risk of primary open angle glaucoma [58]. While the use of clinically relevant doses of systemically administered statins for the management of glaucoma may be dose-limited due to anatomical restrictions (the TM is avascular), topical administration of a selective ROCK inhibitor SNJ-1656 (an ophthalmic solution of Y-39983) is aggressively being explored in human clinical trials as a potentially exciting novel therapeutic agent for the management of elevated IOP associated with some forms of glaucoma [24]. These and other studies continue to generate considerable interest in evaluating the role of isoprenoids as potential therapeutic targets for the management of not only vision-related disorders but a wide variety of neurological diseases, including Alzheimer's disease [36–41].

In conclusion, we show that post-translational prenylation with geranylgeranyl diterpenes selectively facilitates the expression, membrane translocation, functional activation, and turnover of newly synthesized Rho proteins. Geranylgeranyl prenylation represents a novel mechanism by which active Rho proteins are targeted to the 20*S* proteasome for degradation in human TM cells. An isoprenoid-dependent mechanism governing Rho G-protein life cycle exists in human TM cells and most likely represents a general mechanism used by mammalian cells to regulate Rho-dependent cell signaling.

Acknowledgments This work was supported, in part, by grants from the Department of Veterans Affairs (C3638R and B3756-F (EBS), Pre-Doctoral Associated Health Rehabilitation Research Fellowship and C7506M (CVZ)), the Illinois Society for the Prevention of Blindness, the Midwest Eye Banks, and the Richard A. Peritt Charitable Foundation.

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