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Effects of Lovastatin on Rho Isoform Expression, Activity, and Association with Guanine Nucleotide Dissociation Inhibitors

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Abstract

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC1.1.1.88) inhibitors (statins) reduce cholesterol synthesis and prevent cardiovascular disease; they can also inhibit prenylation of Ras and Rho proteins, and have anti-neoplastic effects. Rho proteins cycle between an active, GTP-bound, and an inactive, GDP-bound form, and Rho prenylation is important for Rho's interaction with upstream regulators and downstream effectors, but the effects of statins on Rho signaling are incompletely understood. We found that the HMG-CoA reductase inhibitor lovastatin markedly induced the expression of RhoA, B, and C in human erythroleukemia (HEL) cells. The drug increased RhoA and C only in their unprenylated forms, but it increased both prenylated and unprenylated RhoB and did not significantly affect N- and K-Ras prenylation, suggesting that it inhibited geranylgeranylation more efficiently than farnesylation. Quantitative analysis of nucleotides bound to Rho demonstrated a 3.7-fold increase in Rho·GTP and a similar increase in Rho·GDP in lovastatin-treated cells, leaving the fraction of Rho in the active, GTP-bound form constant at 5.8%. Lovastatin reduced Rho association with Rho guanine dissociation inhibitor (RhoGDI)- α and - β , and prenylation-deficient Rho mutants did not associate with RhoGDI. siRNA inhibition of RhoGDI α expression increased Rho·GTP, suggesting that decreased Rho/RhoGDI α association explained an increase in unprenylated Rho·GTP in lovastatin-treated cells. Unprenylated Rho A, B, and C were partly functional in activating serum response element-dependent transcription. In conclusion, we quantified effects of lovastatin on RhoA, B, and C isoforms, and provide a molecular mechanism whereby statins cause accumulation of unprenylated Rho·GTP.

Keywords

HMG-CoA reductase inhibitor; Prenylation; Rho isoforms; Rho activity; Rho guanine nucleotide dissociation inhibitor; Leukemia cells

INTRODUCTION

Rho GTPases (Rho, Rac, and CDC42) regulate cell growth and apoptosis, and cytoskeletal dynamics involved in cell motility, invasion, and metastasis [1]. Rho proteins cycle between an inactive, GDP-bound and an activated, GTP-bound state; they are activated by guanine

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nucleotide exchange factors (GEFs), and inactivated by GTPase-activating proteins (GAPs). Rho-GDP is mainly cytoplasmic and associated with GDP dissociation inhibitors (GDIs), whereas Rho-GTP is thought to interact with effector proteins at the plasma membrane [2]. Three highly related (85% amino acid identity) Rho isoforms, RhoA, B, and C, appear to interact with the same GEFs and share some of the same effector proteins, but show clear functional differences. In fibroblasts and cancer cells, RhoA and C are predominantly *pro*-proliferative and *anti*-apoptotic, while RhoB is *anti*-proliferative and *pro*-apoptotic [1]. RhoA is required for Ras-induced cellular transformation, and shows increased activation in Ras-transformed cells [1,3]. Activating Rho mutations have not been reported in human malignancies, but several Rho-regulatory proteins were found in screens for transforming genes, and in leukemia-associated chromosomal translocations [1]. In various human cancers, RhoA or C overexpression is associated with a more aggressive phenotype [1].

Rho proteins are post-translationally modified by an isoprenoid group--RhoA and C exclusively by a geranyl-geranyl group, but RhoB by either a geranyl-geranyl or a farnesyl group [4]. Prenylation of Rho proteins is essential for proper membrane localization, and appears to be required for most, but not all, known Rho functions [5–8]. HMG-CoA reductase (EC1.1.1.88) inhibitors (statins) reduce the synthesis of mevalonate, the precursor of cholesterol, and of farnesyl and geranyl-geranyl isoprenoids [9]. In addition to lowering serum cholesterol levels, statins exert “pleiotropic” effects such as reducing vascular inflammation, and improving endothelial function [10]. At clinically achievable concentrations, statins also induce growth arrest and apoptosis in leukemic and other malignant cells, and promising anti-tumor activities have been observed in animal models and early clinical trials [9,11]. It has been proposed that many of these pleiotropic effects are mediated by statins inhibiting Ras and Rho prenylation [10].

Since GEFs activate prenylated Rho in vitro much more efficiently than unprenylated Rho, statins are generally assumed to block Rho activation [5]. However, in vitro studies suggest that the interaction of Rho with GAPs and GDIs may also be affected by Rho’s prenylation state, and in vitro studies may not predict the effect of prenylation inhibitors on Rho activity in intact cells. There are conflicting reports in the literature, whether statins increase or decrease Rho-GTP levels in intact cells [12–16]. Therefore, we decided to study the effects of lovastatin on Rho signaling in human erythroleukemia (HEL) cells. We found that lovastatin caused accumulation of unprenylated RhoA, B, and C in the GTP-bound form, and determined the molecular mechanism of the increase in Rho-GTP.

MATERIALS AND METHODS

Materials and DNA Constructs

Antibodies directed against the following proteins were from Santa Cruz Biotechnology: RhoA (sc-418), RhoB (sc-180), RhoC (sc-26480), H-Ras (sc-29), K-Ras (sc-30), actin (sc-8432), RhoGDI α (sc-360), and RhoGDI β (sc-11359). Rho standards were purified from bacteria to >90% homogeneity as judged by PAGE/Coomassie staining, and were quantified by comparison to bovine serum albumin. Glutathione-S-transferase(GST)-tagged rhotekin Rho binding domain (RBD) was purified from bacteria as described previously [3,17]. Vectors encoding Rho A and B with the N-terminal epitope tag EEEEYMPME (EE-tag) were constructed as described [18]; vectors encoding hemagglutinin (HA)-tagged RhoC (wild type and V14) were purchased from the University of Missouri-Rolla cDNA Resource Center. Prenylation-deficient mutants of RhoA (S190), RhoB (S193), and RhoC (S190) were obtained by PCR using antisense primers with the appropriate nucleotide changes; all PCR products were sequenced. Lovastatin lactone (Calbiochem) was converted to the active acid as described [19].

Cell Culture

HEL cells were cultured in RPMI supplemented with 10% fetal bovine serum. For most experiments, mid-log phase cells were diluted to a density of 2×10^5 cells/ml and exposed for 48 h to 10 μ M lovastatin, unless stated otherwise.

Measurement of Rho Activation

Two different methods were used to measure Rho activation; both rely on the Rho binding domain (RBD) of rhotekin to isolate Rho-GTP. To determine the activation state of each Rho isoform separately, we performed the assay as described by Ren et al. [17], assessing the amount of GTP-bound RhoA, B, or C by Western blotting. To quantify absolute amounts of GTP and GTP+GDP bound to Rho, we employed a coupled enzymatic assay using nucleoside diphosphate kinase and luciferase as described previously [3,18].

Triton X-114 Phase Separation

Rho proteins were analyzed for isoprenylation by Triton X-114 partition [20]. Cells were lysed in ice-cold buffer containing 1% Triton X-114, and after a 2 min centrifugation, the supernatant was warmed to 30°C for 1 min, and micelles formed were pelleted by centrifugation at room temperature. The aqueous supernatant was cooled to 4°C, and adjusted to 1% Triton X-114; the detergent pellet was diluted to the same volume as the aqueous phase using lysis buffer without detergent. Equal volumes of the two fractions were analyzed by SDS-PAGE/Western blotting [18], or were incubated with GST-rhotekin-RBD for measuring absolute amounts of GTP and GDP+GTP as described above [3].

DNA Transfection, Co-immunoprecipitation, and Reporter Gene Assays

HEL cells were transfected using either the Nucleofector™ (Amaxa) protocol (program X-005, solution V), or using DMRIE-C™ transfection reagent (Invitrogen). Human embryonal kidney (293T) cells were transfected by calcium phosphate precipitation. Cells were lysed by Dounce-homogenization, centrifuged, and supernatants were subjected to immunoprecipitation. Luciferase and β -galactosidase reporter gene activities were measured as described previously [18].

siRNA Transfection Experiments

The siRNAs for green fluorescent protein (GFP) was described previously [21]. To silence RhoB expression, we employed Smartpool siRNA reagent M-008395-0005 from Dharmacon. The siRNA for RhoGDI α was directed against the target sequence 5'-CAGGAAAGGCGUCAAGAUUGA-3' [22]. siRNAs were introduced into HEL cells using the Nucleofector™ protocol (Amaxa, program T-016, solution V).

Data Analysis

Bar graphs represent the mean $\pm$ S.D. of at least three independent experiments; Western blots represent typical experiments reproduced at least three times with similar results. We employed the Student's *t* test for comparison of two groups, and ANOVA for multiple comparisons; differences were considered significant at $p < 0.05$.

RESULTS

Lovastatin Increases Expression of Unprenylated RhoA, B, and C in HEL Cells

Treating HEL cells for 48 h with increasing concentrations of lovastatin from 1 to 10 μ M progressively increased the amount of RhoA, B, and C (Fig. 1a, lanes 1–4). RhoA and B antibodies were about equally sensitive in detecting their respective proteins, and more

sensitive than the RhoC antibody (Fig. 1a, lanes 5–7; antibody specificity is shown in lanes 8–10. Please note that 5, 150, or 100 μ g of cellular protein were loaded per lane to generate the blots for RhoA, B, and C, respectively). Taking into consideration the amounts of cell protein used to generate the blots in panel a, we estimate that RhoA constitutes >95% of the three Rho proteins in untreated HEL cells, and that it increased several-fold in cells treated with 10 μ M lovastatin. RhoB and C were below the limits of detection in untreated cells, but constituted about 5 and 10% of Rho proteins, respectively, in lovastatin-treated cells. The band marked with an arrowhead on the RhoB blot represents a cross-reacting protein that migrates with a slightly higher molecular mass than RhoB. To further demonstrate specificity of the RhoB antibody, we treated HEL cells with siRNA oligoribonucleotides targeting RhoB mRNA to prevent RhoB induction by lovastatin. As shown in Fig. 1b, the band representing RhoB was induced by lovastatin in cells treated with a control siRNA targeted at green fluorescent protein (GFP), but it was abolished in RhoB siRNA-treated cells; in contrast, the non-specific band (marked again with an arrowhead) was not affected by the RhoB siRNA.

To quantify Rho prenylation, we extracted cells in the low cloud-point detergent Triton X-114. This allows separating extracts into an aqueous and detergent phase, and previous work showed that unprenylated Ras and Rho proteins partition into the aqueous phase, while prenylated proteins partition into the detergent phase [7,20]. In the absence of lovastatin, RhoA was largely in the detergent phase (Fig. 1c), and RhoB and C were undetectable (the cross-reacting protein recognized by the RhoB antibody is again marked with an arrowhead and partitioned in the aqueous phase). Treating HEL cells for 48 h with 10 μ M lovastatin caused almost all RhoA, B, and C to partition into the aqueous phase, indicating nearly complete inhibition of Rho prenylation (Fig. 1c, left half of panel). The phase separation appeared efficient without loss of cellular proteins, because similar amounts of N- and K-Ras, which are farnesylated, were found in the detergent phase and total cell lysate, and similar amounts of α -actin, which is not prenylated, were in the aqueous phase and lysate (Fig. 1c, lower three panels). From 1–10 μ M lovastatin, we found a progressive shift of RhoA into the aqueous phase, and Rho C appeared exclusively in the aqueous phase (Fig. 1c, right half of panel). RhoB appeared predominantly in the aqueous phase, but a significant amount of RhoB was found in the detergent phase, which may represent farnesylated RhoB (Fig. 1c; see Discussion). Lovastatin did not shift N- and K-Ras into the aqueous phase, suggesting that protein farnesylation was less affected than protein geranylgeranylation up to 10 μ M lovastatin.

To determine whether the accumulation of Rho proteins in lovastatin-treated HEL cells required de novo protein synthesis, we pre-incubated cells for 1 h with the protein synthesis inhibitor cycloheximide prior to the addition of lovastatin. Cycloheximide completely prevented the lovastatin-induced increase in Rho protein, indicating that the accumulation of Rho in lovastatin-treated cells was due to new protein synthesis (Fig. 1d shows results for RhoA and B).

Thus, lovastatin markedly increased the amount of unprenylated RhoA, B, and C in HEL cells, without affecting expression or prenylation of N- and K-Ras. Similar results were obtained with mevastatin (data not shown).

Lovastatin Increases the Amount of GTP-Bound RhoA, B, and C in HEL Cells

Because prenylation is required for Rho proteins to interact with both their activators and inhibitors [5,23,24], an increase in unprenylated RhoA, B, and C could either increase or decrease the amount of the proteins in the activated GTP-bound state. Using a Rhotekin pull-down assay of GTP-bound Rho proteins, we found a striking increase in the amount of activated, GTP-bound RhoA, B, and C in HEL cells treated with 10 μ M lovastatin (Fig. 2, lanes 2–5).

To determine if lovastatin increased the percent of Rho proteins in the activated GTP-bound state [i.e., $\text{Rho-GTP}/(\text{Rho-GTP} + \text{Rho-GDP}) \times 100$], we used a quantitative assay that measures Rho-GTP, and the sum of Rho-GTP plus Rho-GDP [18]. We found a 3.7-fold increase in the absolute amount of Rho-GTP (Fig. 3a, inset), but the percent activation of Rho proteins remained constant at 5.8% after lovastatin treatment, indicating a proportional increase in both Rho-GTP and Rho-GDP (Fig. 3a). This method does not distinguish among GTP-bound RhoA, B, or C, but the measured increase in GTP-bound Rho occurred largely on RhoA, because RhoA constituted > 95% of Rho proteins in untreated and > 85% in lovastatin-treated cells.

Since prenylation affects the interaction of Rho with its regulatory proteins *in vitro*, the activation states of prenylated and unprenylated Rho could be different. To address this question, we analyzed Rho isolated from the aqueous and detergent phases of Triton X-114-extracted cells, realizing that the absolute amounts of nucleotide-bound Rho in the aqueous and detergent phase cannot be directly compared (because the two phases have different protein concentrations). However, the percent activation can be compared, and, in untreated cells, we found a trend towards higher Rho activation in Rho isolated from the detergent phase than from the aqueous phase: $9.5 \pm 3.2\%$ compared to $5.3 \pm 1.1\%$, respectively (Fig. 3b). In lovastatin-treated cells, Rho activation in the aqueous phase was $6.1 \pm 1.5\%$ (Fig. 3b, left panel), which is similar to that of Rho isolated from whole cell extracts. We could not determine the level of Rho activation in the detergent phase of lovastatin-treated cells, because of the extremely small amount of prenylated Rho in this condition.

In conclusion, lovastatin increased the amount of activated, GTP-bound RhoA, B, and C, but the proteins were unprenylated. Similar results were obtained with mevastatin (data not shown). The amount of GDP-bound Rho, at least for RhoA, increased proportionately with the amount of GTP-bound Rho, leaving the percent activation unchanged.

Lovastatin Decreases Rho Association with RhoGDI

Under basal conditions, GDP-bound Rho proteins are thought to be associated primarily with RhoGDIs, and, after cellular stimulation, they dissociate from the RhoGDI, and are activated by a GEF-mediated exchange of GTP for GDP [2]. To study the mechanism of the lovastatin-induced increase in Rho-GTP, we assessed Rho/RhoGDI α association, and found about 70% less RhoA associated with RhoGDI α in lovastatin-treated HEL cells compared to untreated cells (Fig. 4a, compare lanes 5 and 6). This finding was particularly striking considering the large increase in Rho-GDP (Fig. 3a) and total RhoA (Fig. 4a, lane 2) that occurred in cells treated with lovastatin. Co-immunoprecipitation of RhoA and RhoGDI α increased proportionally with increasing amounts of cell extract, indicating quantitative recovery within the limits of the assay (Fig. 4a, lanes 7–9). Thus, as observed *in vitro* with purified proteins [24], unprenylated Rho showed decreased association with RhoGDI α *in vivo*, and the decreased association could explain the increase in Rho-GTP.

The interaction between RhoGDI and Rho is mediated by lipid-protein and protein-protein interactions, and regulated by proteins competing with RhoGDI for Rho binding [2]. To examine the association of RhoB and C with RhoGDI α , and to determine if the prenylation state of Rho alone explains the decreased Rho/RhoGDI association observed in lovastatin-treated cells, we transfected 293T cells with epitope-tagged wild type and prenylation-deficient Rho constructs. The prenylation-deficient Rho proteins have a serine replacing the C-terminal cysteine in the CAAX box, which prevents prenylation as well as subsequent post-translational modifications [4]. When wild type and mutant RhoA^{S190} or RhoC^{S190} were isolated by immunoprecipitation, RhoGDI α was present in the immunoprecipitates of the wild type, but not the non-prenylated Rho proteins (Fig. 4b). No interaction of RhoGDI α with either wild type or non-prenylated RhoB could be detected, although large amounts of RhoB were present

in the immunoprecipitates. This finding is consistent with previous work showing that RhoA, but not RhoB, interacts with fluorescently-labeled RhoGDI α in living cells [25].

To determine if RhoB associated with RhoGDI β (LyGDI), a hematopoietic cell-specific GDI that binds RhoA, Rac, and CDC42, albeit less efficiently than RhoGDI α [26], we transfected HEL cells with epitope-tagged Rho constructs, and examined anti-epitope immunoprecipitates for RhoGDI β . Small amounts of RhoGDI β were associated with wild type, but not unprenylated RhoA; no RhoGDI β was detectable in the immunoprecipitates containing wild type or unprenylated RhoB (data not shown).

Thus, only prenylated, but not unprenylated RhoA and RhoC associated with RhoGDI α (and RhoGDI β for RhoA) in intact cells. RhoB may be associated with RhoGDI γ , a recently described GDI which is found in a detergent-insoluble cytoskeletal fraction and interacts specifically with post-translationally modified RhoB in vitro [27]. The lack of available RhoGDI γ antibodies and poor protein solubility precluded us from studying this interaction in intact cells.

RhoGDI α Knock-down Increases the Amount of Rho-GTP

To determine whether changing Rho association with RhoGDI α is sufficient to change Rho GTP-loading, we used an siRNA strategy to reduce RhoGDI α expression. Transfecting HEL cells with a RhoGDI α -specific siRNA substantially reduced RhoGDI α compared to cells transfected with a control siRNA targeted against GFP (Fig. 5a, top panel). The RhoGDI α siRNA reduced RhoA, whereas it had no effect on K-Ras, Rap1A, or tubulin (Fig. 5a; tubulin not shown). These findings suggest that lack of RhoGDI α binding to prenylated RhoA may render RhoA unstable, similar to Rac1 and CDC42, which are destabilized in RhoGDI α -deficient cells due to increased proteolysis [22]. In contrast, *unprenylated* RhoA is less susceptible to proteolysis than prenylated RhoA [28], which can explain its accumulation in lovastatin-treated cells, despite decreased association with RhoGDI α .

To determine the effect of decreased Rho/RhoGDI α association on Rho-GTP levels, we measured GTP and GTP+GDP bound to Rho in HEL cells transfected with RhoGDI α siRNA, or control siRNA. Total GTP+GDP bound to Rho decreased by about 20% in cells transfected with the RhoGDI α -specific siRNA, compared to control siRNA-transfected cells (Fig. 5b); this agrees with the decreased RhoA observed on Western blots (Fig. 5a). The amount of Rho-GTP increased significantly in the RhoGDI α -deficient cells, increasing the percent of Rho in the activated, GTP-bound state from $3.9 \pm 0.6\%$ to $8.3 \pm 1.1\%$ in control versus RhoGDI α siRNA-transfected cells, respectively (Fig. 5b). Thus, decreased association between Rho and RhoGDI α leads to increased Rho-GTP levels. These results also suggest that RhoGDI α is a major RhoA-binding GDI in HEL cells.

Unprenylated RhoA, B, and C Exhibit Partial Transcriptional Activity

Rho activation of a serum response factor/megakaryoblastic leukemia 1 (MKL1) complex is required for transcriptional regulation of genes controlled by serum response elements (SREs); a t(1;22) translocation in acute megakaryoblastic leukemia generates a MKL1 fusion protein with increased transcriptional activity, suggesting that activation of SRE-dependent genes may contribute to leukemogenesis [29]. To compare the effects of prenylated versus unprenylated Rho-GTP on SRE-dependent transcription, we used constitutively-active, GTP-bound forms of RhoA, B, and C with valine substituted for glycine 14; the unprenylated proteins additionally contained serine substituted for cysteine 190, or 193 in the case of RhoB [4,18].

RhoA^{V14} stimulated transcription from a SRE-luciferase reporter about 25-fold in the absence of lovastatin, and eight-fold in the presence of lovastatin; the unprenylated double mutant

RhoA^{V14/S190} stimulated transcription about five-fold in the absence of lovastatin (Fig. 6a). Similarly, transcriptional effects of RhoB^{V14} and RhoC^{V14} were reduced by lovastatin, and unprenylated RhoB^{V14/S193} and RhoC^{V14/S190} had less activity compared to their prenylated counterparts, but similar activities as those observed with RhoB^{V14} and RhoC^{V14} in lovastatin-treated cells. Expression of the prenylated and unprenylated Rho constructs was comparable, and did not explain the differences in transcriptional activation (Fig. 6b). Thus, unprenylated Rho-GTP exhibited partial transcriptional activity.

DISCUSSION

Statins were originally developed as inhibitors of cholesterol synthesis, but they have pleiotropic, cholesterol-independent effects. Many of these latter effects have been attributed to inhibition of Ras and Rho signaling, and a potential role for statins in cancer prevention and therapy has been suggested [9,30]. We found that prenylation of RhoA and C was almost completely inhibited in HEL cells treated with 10 μ M lovastatin, whereas N- and K-Ras farnesylation was not inhibited, suggesting that farnesylation was less affected than geranyl-geranylation. In other cell types, 10–25 μ M lovastatin decreased RhoA and Rac1 membrane association without affecting membrane-bound Ras, suggesting a differential effect of statins on prenylation of Rho and Ras proteins [31,32]. This may be because farnesyl transferase (EC2.5.1.58) has a K_m for farnesyl diphosphate of ≤ 5 nM, whereas geranyl-geranyl diphosphate synthase (EC2.5.1.29, the enzyme which produces the substrate for geranyl-geranyl transferase, EC2.5.1.59) has a K_m of about 1 μ M for the same substrate [30]. Thus, when intracellular isoprenoids are reduced by statins, geranyl-geranylation would be more severely affected than farnesylation. These data likely explain why a significant fraction of RhoB remained prenylated in lovastatin-treated HEL cells, because, unlike RhoA and C, RhoB is a substrate for farnesyl transferase, although it is not as efficiently farnesylated as Ras [33].

Lovastatin increased RhoA protein levels several-fold, and increased RhoB and C to an even greater extent, but total amounts of RhoB and C remained less than one-tenth that of RhoA. Experiments with cycloheximide showed that the statin-induced Rho accumulation required de novo protein synthesis. In addition, knock-down of RhoB mRNA with specific siRNA oligoribonucleotides prevented lovastatin-induced RhoB accumulation, suggesting a requirement for de novo mRNA and/or protein synthesis (similarly, transfection of RhoA- or RhoC-specific siRNAs prevented RhoA or RhoC accumulation, respectively; unpublished results). These results are in agreement with the findings of others who attributed the statin-induced increases in RhoA and B protein to increased mRNA transcription, increased de novo protein synthesis, and decreased degradation of the unprenylated proteins [28], but we are unaware of previous studies quantifying statin effects on RhoA, B, and C in the same cell type.

When assessed in vitro, Rho prenylation affects RhoA activation by GEFs, RhoA association with RhoGDI, and Rho de-activation by Rho GTPase-activating proteins (RhoGAPs) [5,23, 34]. Therefore, it is difficult to predict how inhibition of Rho prenylation in vivo will affect the activation state of Rho. The prevailing view is that statins inhibit Rho signaling by preventing Rho membrane association, and block agonist-induced Rho activation [12–14,30, 35,36]. These studies generally examined short-term (< 6 h) effects of statins on acute, GEF-mediated Rho activation. In contrast, we examined longer-term (24–48 h) effects of statins, and found that lovastatin induced a several-fold increase in total Rho-GTP, and that the increase in GTP occurred on unprenylated Rho. Longer-term statin treatment of cardiac myofibroblasts and endothelial cells increased Rho-GTP and Rac-GTP levels, respectively, but the mechanism of this increase was not examined [16,37]. While this work was in progress, Cordle et al. [15] reported that treating THP-1 monocytic cells with statins for 18 h increased Rac- and Rho-GTP levels, and that the effect on Rac could be prevented when cells were incubated with

mevalonate or geranyl-geranylpyrophosphate to bypass the statin block and reconstitute Rac prenylation.

We propose that lovastatin increases Rho-GTP, at least in part, by decreasing Rho binding to RhoGDI. In vitro, binding of unprenylated Rho to RhoGDI is weak [24], and we showed that Rho prenylation is required for formation of stable Rho-RhoGDI complexes in intact cells. Using siRNA-mediated knock-down of RhoGDI α , we also demonstrated that decreased Rho-RhoGDI association is sufficient to increase Rho-GTP levels. Phosphorylation of RhoGDI can increase Rho activation, possibly by dissociating the Rho-RhoGDI complex [2]. Our finding of decreased RhoA protein in RhoGDI α -deficient cells suggests that RhoGDI binding to prenylated Rho stabilizes Rho and protects it from proteolysis, as occurs for Rac [22]. Increased Rho in lovastatin-treated cells required de novo protein synthesis leading to the accretion of unprenylated Rho proteins, which did not bind to RhoGDIs; unprenylated RhoA and B have been shown previously to be more stable than their prenylated counterparts [28], explaining their accumulation in the absence of RhoGDI binding. Increased accumulation of unprenylated Rho-GTP may be due to spontaneous GTP exchange in the absence of RhoGDI binding; alternatively, GEFs could act on unprenylated Rho, although this occurs only to a limited extent in vitro [34]. Whether RhoGAP acceleration of Rho-GTP hydrolysis is influenced by Rho prenylation is controversial [5,23].

It is generally assumed that unprenylated Rho proteins cannot interact with down-stream effectors [6]. In yeast, the unprenylated form of Ras2 has more than 100-fold lower affinity for its effector adenylate cyclase compared to the prenylated form [38], but in mammalian cells, unprenylated Ras may form inactive complexes with its effectors Raf-kinase [39]. We found that unprenylated RhoA, B, and C had some transcriptional activity in HEL cells, albeit significantly less than their prenylated counterparts. Thus, unprenylated Rho appears to interact with effectors that stimulate SRE-dependent transcription, which may include Rho-kinase and protein kinase C-related kinases [18], but the interaction is less productive than with prenylated Rho.

In conclusion, we found that lovastatin markedly increased GTP-bound, unprenylated RhoA, B, and C in HEL cells, and demonstrated that the molecular mechanism of the increase involved lack of association of unprenylated Rho with RhoGDI. The functionality of unprenylated Rho-GTP may depend on the repertoire of Rho effectors present in specific cell types, and has important implications for the pleiotropic, cholesterol-independent effects of statins.

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The abbreviations used are

GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor

GFP	green fluorescent protein
GST	glutathione-S-transferase
HEL cells	human erythroleukemia cells
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
RBD	Rho binding domain

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Figure 1. Lovastatin Increases Expression of Unprenylated Rho A, B, and C in HEL Cells. Panel a HEL cells were treated for 48 h with increasing concentrations of lovastatin (lanes 1–4); 5, 150, or 100 μ g (for RhoA, B, or C, respectively) of extracted cellular protein was analyzed by SDS-PAGE/Western blotting using antibodies specific for each Rho isoform. Purified RhoA, B, or C (4, 8, and 16 ng) were analyzed in parallel (lanes 5–7). To determine antibody specificity, 40 ng of purified Rho A, B, or C were loaded in lanes 8–10 as indicated. The arrowhead on this and subsequent RhoB blots denotes a protein of slightly higher molecular weight than RhoB that cross-reacts with the RhoB antibody. **Panel b:** HEL cells were transfected with siRNA oligoribonucleotides specific for RhoB (lanes 1 and 2) or with control siRNA specific for GFP (lanes 3 and 4), and were cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 3 μ M lovastatin for 48h. Cell lysates were analyzed by Western

blotting using the RhoB-specific antibody described in panel a. **Panel c:** HEL cells were cultured for 48 h in the absence (control, lanes 1–3 and 7–8) or presence of lovastatin (10 μ M lovastatin in lanes 4–6; lovastatin concentration as indicated in lanes 9–14). Cells were extracted in 1% Triton-X-114, and total cell lysates (T) were separated into an aqueous supernatant (A), and a detergent-containing pellet (D). Aliquots of total cell lysate, aqueous supernatant (containing unprenylated proteins), and detergent pellet (containing prenylated proteins) were analyzed by SDS-PAGE/Western blotting. The actin blot demonstrated minimal protein loss during extraction, and no contamination of the detergent pellet with proteins from the aqueous phase. **Panel d:** HEL cells were pre-treated with 2 μ g/ml of cycloheximide (CHX, lanes 1 and 2) or left untreated (lanes 3 and 4) prior to the addition of 10 μ M lovastatin for 24 h (lanes 2 and 4). Whole cell lysates were analyzed by Western blotting as described in panel a.

Figure 2. Lovastatin Increases the Amount of GTP-bound RhoA, B, and C in HEL Cells
 HEL cells were cultured for 48 h in the absence (lanes 2, 3, 6, and 7) or presence (lanes 4, 5, 8, and 9) of 10 μ M lovastatin. Cell lysates were incubated with a rhotekin-RBD-GST-fusion protein bound to glutathione agarose beads to isolate GTP-bound Rho proteins. Beads coated with GST alone served as a control (GST, lane 1). GTP-bound RhoA, B, or C associated with the rhotekin-RBD (RBD pulldown, lanes 2–5) and total RhoA, B, or C present in 3% of the whole cell lysate (input, lanes 6–9) were assessed by Western blotting. As described in the legend of Fig. 1, the arrowhead denotes a cross-reacting protein detected by the RhoB antibody.

Figure 3. Quantification of GTP and GDP Bound to Prenylated versus Unprenylated Rho. Panel a

HEL cells were cultured and extracted as described in Fig. 2, and the amounts of total nucleotides (GTP + GDP, striped bars) or GTP (filled bars) bound to Rho in unfractionated cell lysates were measured enzymatically. The percent of Rho in the active, GTP-bound form is indicated. The inset shows GTP-bound Rho on an expanded scale. **Panel b:** HEL cells were cultured in the absence or presence of 10 μ M lovastatin and extracted in Triton-X-114. Unprenylated Rho proteins present in the aqueous phase (left panel) and prenylated Rho proteins present in the detergent phase (right panel) were subjected to an RBD pulldown assay; the amounts of total nucleotides (GTP + GDP, striped bars) or GTP (filled bars) bound to Rho were measured enzymatically. The inset in the left panel shows GTP-bound Rho on an expanded scale. The amount of Rho present in the detergent phase in lovastatin-treated cells was below the limit of detection. The detergent phase had a lower protein concentration than the aqueous phase, making the denominator for the amount of nucleotides bound to Rho different between the two phases. In panels a and b, the comparison between control and

lovastatin-treated cells showed a significant difference for both total nucleotides (GTP+GDP) and GTP bound to Rho ($p<0.05$).

Figure 4. Lovastatin Decreases RhoA Association with RhoGDI. **Panel a** HEL cells were cultured for 48 h in the absence (lanes 1, 3, 5, and 7–9) or presence of 10 μ M lovastatin (lanes 2, 4, and 6), and cell extracts were subjected to immunoprecipitation with a RhoGDI α -specific antibody (lanes 5–9) or control IgG (lanes 3 and 4). Immunoprecipitates were analyzed by Western blotting with an antibody specific for RhoA (upper panels); blots were stripped and re-probed with an antibody for RhoGDI α (lower panels). Input cell lysates were analyzed in parallel (lanes 1 and 2; 3% of total lysate). The amount of RhoA co-immunoprecipitated with RhoGDI α was proportional to the amount of RhoGDI in the immunoprecipitates as demonstrated in the right half of the panel: lanes 7, 8, and 9 show 100, 30, and 10% of the cell lysate, respectively, subjected to immunoprecipitation. **Panel b:** Human embryonal kidney (293T) cells were transfected with empty vector (E.V., lanes 1, 4, and 7), epitope-tagged wild type Rho (WT, lane 2 for RhoA, lane 5 for RhoB, and lane 8 for RhoC) or prenylation-deficient, mutant Rho constructs (lane 3 for RhoA^{S190}, lane 6 for RhoB^{S193}, and lane 9 for RhoC^{S190}). Cell lysates were subjected to immunoprecipitation using an epitope-specific antibody, and precipitates were analyzed by Western blotting using a RhoGDI α antibody (upper panel). Blots were stripped and re-probed with antibodies for RhoA (lanes 1–3), RhoB (lanes 4–6), or RhoC (lanes 7–9).

Figure 5. Decreased Rho/Rho-GDI Binding Causes Increased Rho-GTP. Panel a HEL cells were transfected with siRNA oligoribonucleotides specific for RhoGDI α (lanes 3 and 4) or with control siRNA specific for GFP (lanes 1 and 2). Cells were extracted 48 h later, and the extracts were subjected to SDS-PAGE/Western blotting using antibodies specific for RhoGDI α (top panel), RhoA (second panel), K-Ras (third panel), and Rap1A (lower panel). **Panel b:** HEL cells were transfected as described in panel c; after 48 h, cells were extracted and total nucleotides (GTP+GDP, striped bars) or GTP (filled bars) bound to Rho were measured as described in the legend to Fig. 3a. The percent of total Rho in the active, GTP-bound state is indicated for each condition, and the inset shows GTP-bound Rho on an expanded scale. Comparing cells treated with control versus RhoGDI-specific siRNA yielded a significant difference in both total nucleotides (GTP+GDP) and GTP bound to Rho ($p < 0.05$).

Figure 6. Unprenylated RhoA, B, and C Exhibit Partial Functionality in Regulating SRE-Dependent Gene Transcription. Panel a

HEL cells were transfected with a luciferase reporter gene under control of a serum response-element-dependent promoter (pSRE-Luc), and were co-transfected with either empty vector, or the indicated RhoA, B, or C expression vectors. All Rho constructs contained valine substituted for glycine 14, rendering them constitutively active (GTP-bound); the unprenylated versions additionally contained serine substituted for cysteine 190 (RhoA and C) or 193 (RhoB). Cells were cultured in the absence (gray and open bars) or presence (black bars) of 10 μ M lovastatin for 48 h. Gray and black bars represent cells transfected with single mutant Rho^{V14}; open bars represent cells transfected with double mutant RhoA^{V14/S190}, RhoB^{V14/S193}, and RhoC^{V14/S190}. Luciferase activities were normalized to the activity found in untreated cells transfected with pSRE-Luc plus empty vector. **Panel b:** HEL cells were transfected with empty vector (lane 1), or epitope-tagged Rho constructs as described in panel a (lane 2 for single mutant Rho^{V14}, lane 3 for double mutant RhoA^{V14/S190}, RhoB^{V14/S193}, and RhoC^{V14/S190}). Cell lysates were analyzed by Western blotting with anti-epitope-specific antibodies.