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J. Biol. Chem. 2002, 277:49389-49396.
doi: 10.1074/jbc.M204049200 originally published online October 15, 2002

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 51, Issue of December 20, pp. 49389–49396, 2002
Printed in U.S.A.

Isoprenylation of RhoB Is Necessary for Its Degradation

A NOVEL DETERMINANT IN THE COMPLEX REGULATION OF RhoB EXPRESSION BY THE MEVALONATE PATHWAY*

Received for publication, April 25, 2002, and in revised form, September 25, 2002
Published, JBC Papers in Press, October 15, 2002, DOI 10.1074/jbc.M204049200

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Statins improve vascular functions by mechanisms independent from their cholesterol-lowering effect. Rho GTPases are emerging as key targets for the vascular effects of statins. RhoB is a short-lived, early-response inducible protein involved in receptor endocytosis, apoptosis, and gene expression. Here we show that statins regulate RhoB expression by acting at multiple levels. Simvastatin increased RhoB protein levels by 8- to 10-fold. This effect was related to a depletion of isoprenoid intermediates, as deduced from the observation that several metabolites of the cholesterol biosynthetic pathway, namely, mevalonate and geranylgeranyl-pyrophosphate, attenuated simvastatin-induced RhoB up-regulation. Moreover, prenyltransferase inhibitors mimicked simvastatin effect. Cholesterol supplementation did not prevent simvastatin-elicited up-regulation but increased RhoB levels *per se*. Simvastatin moderately augmented RhoB transcript levels, but markedly impaired the degradation of RhoB protein, which accumulated in the cytosol in its non-isoprenylated form. Inhibition of RhoB isoprenylation was apparently required for simvastatin-induced up-regulation, because levels of an isoprenylation-deficient RhoB mutant were not affected by simvastatin. Moreover, this mutant was found to be markedly more stable than the wild-type protein. These results show that RhoB isoprenylation is necessary for rapid turnover of this protein and identify a novel link between the cholesterol biosynthetic pathway and the regulation of G-protein expression.

Statins are a family of drugs widely used in the treatment of hypercholesterolemia. They are competitive inhibitors of hydroxymethylglutaryl-CoA (HMG-CoA)[1] reductase (1), the enzyme catalyzing the rate-limiting step in the synthesis of cholesterol, that is, the conversion of HMG-CoA to mevalonate (Scheme 1). Mevalonate is also the precursor of farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, the isoprenoid lipids involved in the posttranslational modification of a number of proteins, including most G-proteins (2, 3). For this reason, statins have also been used as pharmacological tools to assess the importance of protein isoprenylation in signal transduction (4, 5).

The clinical use of statins has revealed that these drugs promote beneficial effects on cardiovascular functions that do not correlate with their ability to lower serum cholesterol levels (6, 7). Treatment of endothelial cells with statins increases or preserves the expression of endothelial nitric oxide synthase (8, 9), while inhibiting the production of endothelin-1 (10). In addition, antiproliferative and anti-inflammatory effects of statins have been observed in several experimental systems (5, 11–13) and in patients (14, 15). Elucidation of the mechanisms underlying these diverse effects has shown that small G-proteins of the Rho family are critical targets for statins in many systems. Rho GTPases participate in the regulation of numerous cellular functions, including cytoskeletal organization, cell adhesion, smooth muscle contraction, endocytosis, receptor signaling, cell cycle progression, and gene expression (16–19). Rho proteins are posttranslationally modified by isoprenylation at a cysteine residue located near their C-terminal end (20). Inhibition of Rho function by impairment of protein isoprenylation is at the basis of the reported effects of statins on smooth muscle cell proliferation (11), endothelial nitric oxide synthase and inducible nitric oxide synthase expression (8, 21), pre-pro-endothelin-1 transcription (10), and protection against cerebral stroke (22). Evidence accumulating during the last decade indicates that, by limiting mevalonate availability, statins may influence not only the function but also the expression of several G-proteins, including Ras (23), Rab (24), Rap (25), and Rho proteins (26–28). However, the mechanisms responsible for these effects are not fully elucidated.

RhoB is a short-lived protein, the levels of which can be regulated by growth factors and stress signals both transcriptionally and posttranslationally (26, 29). RhoB can be modified either by farnesylation or geranylgeranylation at its C terminus (20), and it has been proposed that its function may depend on the presence and the nature of the isoprenoid moiety (30). Among the reported RhoB functions are the modulation of receptor endocytosis (31), apoptosis (32), and vascular gene expression, including that of pre-pro-endothelin-1 (10) and inducible nitric oxide synthase (33). A detailed knowledge of the effect of statins on Rho protein biology is of primary importance in the context of the therapeutic strategies for hypercholesterolemia and atherosclerosis. In the present study we have ex-

* This work was funded by grants from Ministerio de Ciencia y Tecnología (SAF2000-0149), Fundación Ramón Areces and Merck, Sharp & Dohme, Spain. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of fellowships from Fundación Ramón Areces and Residencia de Estudiantes (Ayuntamiento de Madrid, Spain).

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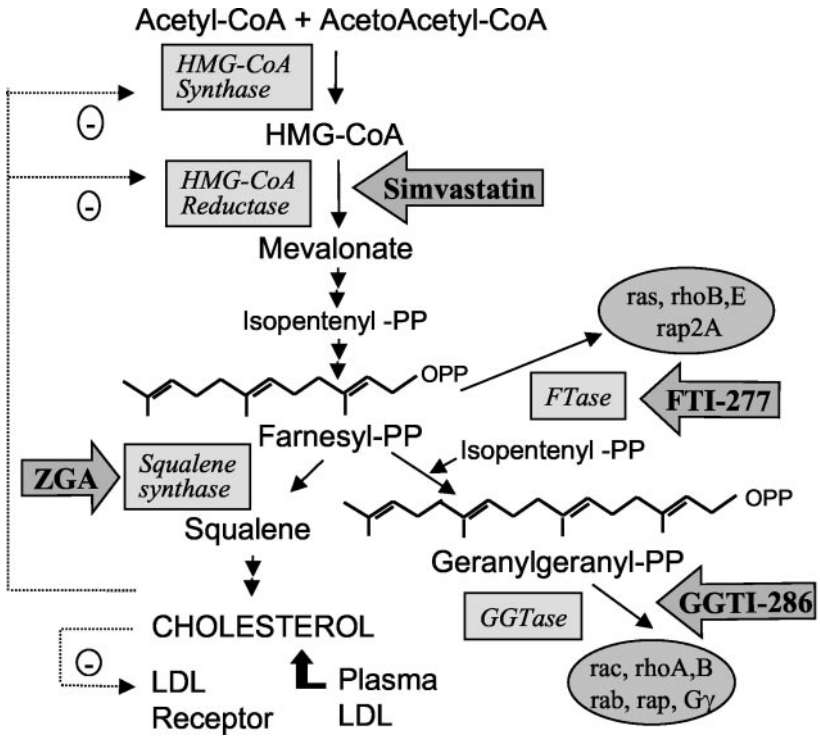
[1] The abbreviations used are: HMG-CoA, hydroxymethylglutaryl-CoA; BAEC, bovine aortic endothelial cells; GGPP, geranylgeranyl-pyrophosphate; GFP, green fluorescent protein; HA, hemagglutinin.



SCHEME 1. **Main intermediates in the cholesterol/isoprenoid biosynthetic pathway and pharmacological tools used to explore it.** Enzymes of this pathway are represented in *shaded rectangles* and the corresponding inhibitors in *arrowheads*. *FTase*, farnesyltransferase; *GGTase*, geranylgeranyltransferase; *ZGA,* zaragocic acid; *LDL*, low density lipoproteins.

plored the effect of statins on RhoB expression and processing in several cell types. Our results show that statins drastically increase the levels of cellular RhoB by mechanisms that involve the regulation of RhoB mRNA by the cholesterol biosynthetic pathway and the impairment of the proteolytic degradation of the non-isoprenylated RhoB protein. This latter observation provides the first evidence for the regulation of protein stability by isoprenylation.

MATERIALS AND METHODS

Materials—Simvastatin, lovastatin, and zaragocic acid were from Merck Sharp and Dohme. Antibodies anti-RhoA (sc-418), anti-RhoB (sc-180), and anti-HA (sc-7392) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pan-Ras-10 (Ab-3) monoclonal antibody was from Oncogene Science (Uniondale, NY). Peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). Deoxycytidine 5′-triphosphate [α-^{32}P]- (3,000 Ci/mmol) was from Amersham, Aylesbury, UK. FTI-277, GGTI-286, toxin B from *Clostridium difficile*, C3 exoenzyme from *Clostridium botulinum*, and the Rho-associated kinase inhibitor Y-27632 were from Calbiochem-Novabiochem (San Diego, CA). All other reagents were of the highest purity available from Sigma.

Cell Culture—Bovine aortic endothelial cells (BAEC) were isolated from thoracic aortas and characterized as previously described (9). Cells were maintained in RPMI 1640 supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere with 5% CO_2. Experiments were performed on confluent monolayers at passages 3–8 made quiescent by serum deprivation for 24 h. Potential toxicity of the reagents used was tested by trypan blue exclusion and by analysis of the cell cycle distribution by flow cytometry (34). The concentrations of simvastatin used throughout this study did not induce apoptosis in BAEC. Mesangial cells were obtained and characterized as reported previously (35). Human umbilical vein endothelial cells were isolated and cultured according to published procedures (36). Mv1Lu, HeLa, and NIH-3T3 cells were from the Animal Cell Culture facility of Centro de Investigaciones Biológicas. Mv1Lu and NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and HeLa cells were cultured in RPMI 1640 plus 10% fetal bovine serum.

SDS-PAGE and Immunoblotting—For SDS-PAGE, BAEC were homogenized by forced passes through a 26½-gauge needle in 10 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM β-mercaptoethanol, containing 2 μg/ml of each of the protease inhibitors leupeptin, aprotinin, and pepstatin. Protein was measured by the BCA method (Pierce, Rockford, IL), and aliquots containing 20 μg of protein were electrophoresed on 15% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were incubated with anti-RhoA (1:1,000), anti-RhoB (1:500), or anti-pan-Ras (1:1,000) antibodies, and the proteins of interest were visualized using an ECL detection system from Amersham Biosciences. Levels of RhoA and B proteins were estimated by image scanning of the ECL exposures. The values obtained were corrected by the band intensities of the Coomassie staining of membranes after the blotting procedure or by the signal given by an antibody against a non-related protein.

Subcellular Fractionation—For obtention of S100 (cytosol) and P100 (membrane) fractions, cells were homogenized in 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, and 0.1 mM dithiothreitol containing protease inhibitors, and lysates were centrifuged at 200,000 $\times$ *g* for 30 min at 4 °C in a Beckman TLA100 rotor.

Determination of RhoB Protein Stability—For this purpose, a previously published method was used (26). After treatment in the absence or presence of simvastatin, cells were incubated with 20 μg/ml cycloheximide, a concentration that effectively blocked synthesis of RhoB protein, and levels of RhoB were analyzed by Western blot at various time points after cycloheximide addition.

RNA Isolation, Northern Blotting, and Hybridization—Total cellular RNA was isolated from BAEC with the guanidinium thiocyanate-phenol-chloroform method and processed as previously reported (10). Equal loading was ensured by ethidium bromide staining. For analysis of RhoB mRNA expression, the full-length rat RhoB cDNA, a gift of Dr. G. C. Prendergast (The Wistar Institute, Philadelphia, PA), was used as a probe. Blots were stripped and rehybridized with a probe for human β-actin. Densitometric analysis was performed using an Agfa StudioStar TPO scanner with the public domain software NIH IMAGE 1.60b5. Results were calculated as the ratio of RhoB/β-actin mRNA levels.

Plasmids and Transfections—The mammalian expression vector encoding the rat RhoB cDNA tagged at the amino terminus with a hemagglutinin epitope (pcDNA3-HA-RhoB) was generously provided by Dr. G.C. Prendergast. The isoprenylation-deficient HA-RhoBC193S mutant was generated from the above plasmid by PCR using primers forward: 5′-CCAGAGAGATCTATGTACCCATACGATGTGCCAGAC-3′ and reverse: 3′-CAGAGCTCTAGATCATAGCACCTTCGAGCAGTTGATG-5′. The DNA fragments encoding wild-type and mutant HA-RhoB were subcloned into the *Bgl*II, *Eco*RI sites of the pEGFP-C1 vector (Clontech). Cells grown on 6-well plates or on 12-mm glass coverslips were transfected using LipofectAMINE Plus reagent (Invitrogen) fol-

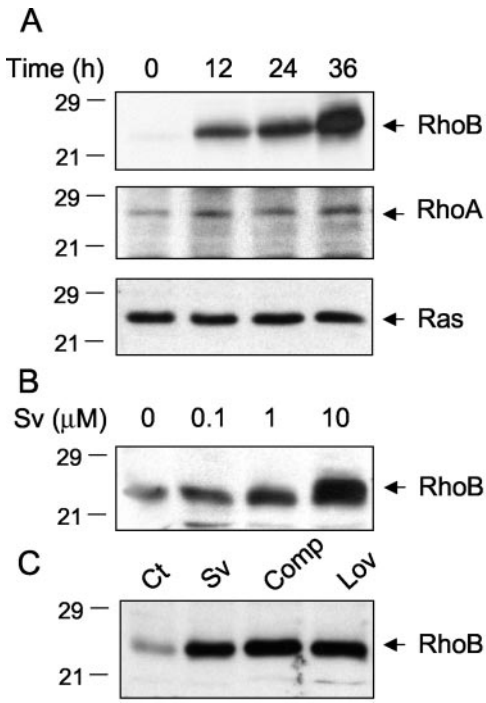


FIG. 1. **Effect of HMG-CoA inhibitors on the abundance of RhoB protein in BAEC.** *A*, BAEC were treated with simvastatin (*Sv*) at 10 μM for the indicated time periods, and the levels of RhoB, RhoA, and Ras proteins were assessed by immunoblot. *B*, BAEC were treated with the indicated concentrations of simvastatin or (*C*) with different HMG-CoA inhibitors at 10 μM for 24 h. Results shown are representative of at least three experiments. *Lov*, lovastatin; *Comp*, compactin.

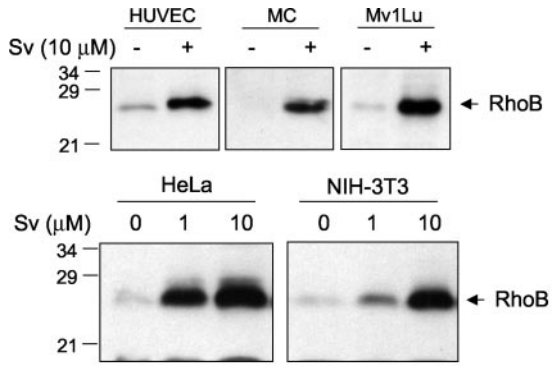


FIG. 2. **Effect of simvastatin on RhoB protein levels in several cell types.** The indicated cell types were treated with simvastatin for 24 h. *HUVEC*, human umbilical vein endothelial cells; *MC*, glomerular mesangial cells; *Mv1Lu*, mink lung epithelial cells; *HeLa*, epitheloid carcinoma cells; *NIH-3T3*, mouse fibroblasts. RhoB levels were assessed by Western blot. Results shown are representative of at least two experiments.

lowing the instructions of the manufacturer. After a 24-h recovery period, cells were exposed to the various agents. The expression of RhoB or of GFP-RhoB proteins was monitored by Western blot or confocal fluorescence microscopy, as indicated. Transfection efficiency was ~80% in NIH-3T3 cells as estimated from fluorescence microspcopy analysis of GFP-RhoB transfected cells.

RESULTS

HMG-CoA Inhibitors Increase RhoB Protein Levels in Vascular Endothelial Cells—Treatment of quiescent BAEC with the HMG-CoA inhibitor simvastatin led to a time- and dose-dependent increase in RhoB protein levels (Fig. 1). An increase in RhoB could be detected after a 24-h treatment with 0.1–1 μM simvastatin, a concentration in the range of that reached in the plasma of patients treated with this drug (37), although the effect was more evident at simvastatin concentrations above 1 μM, which are higher than those seen in humans. Treatment of BAEC with 10 μM simvastatin for 24 h resulted in 6- to 10-fold higher RhoB protein levels. RhoA protein was also more abundant after simvastatin treatment (2- to 3-fold increase). The levels of Ras proteins, detected with a pan-Ras antibody, did not change appreciably under these experimental conditions. A similar effect on RhoB protein levels was brought about by other HMG-CoA reductase inhibitors, namely, lovastatin and compactin (Fig. 1*C*). This phenomenon was not restricted to BAEC, because it could also be evidenced in human umbilical vein endothelial cells and mesangial cells treated with simvastatin (Fig. 2). Simvastatin induced an increase in RhoB levels in all the cell types thus far studied, including 293T and COS-7 cells (data not shown), the epithelial cell lines Mv1Lu and HeLa, and in NIH-3T3 fibroblasts (Fig. 2).

Regulation of RhoB Expression by the Mevalonate Pathway—Statins are potent cholesterol-lowering agents. Therefore we explored whether the effect of simvastatin could be prevented by increasing cholesterol supply. Cholesterol supplementation did not prevent simvastatin-induced RhoB up-regulation in BAEC (Fig. 3*A*, *left*); conversely, a moderate up-regulation of RhoB protein levels could be detected in cholesterol-supplemented cells (1.6 ± 0.12-fold increase with respect to control levels, $n = 4$). Because both simvastatin and cholesterol reduce the flux through the cholesterol biosynthetic pathway, these observations suggest that a down-regulation of this pathway or the depletion of some intermediate(s) may be involved in the effect of simvastatin. In keeping with this, treatment of BAEC with zaragocic acid, an inhibitor of squalene synthase, expected to increase the activity of the pathway (38), moderately reduced RhoB protein levels (20–30% reduction, $n = 3$, not shown). Incubation of BAEC in the presence of mevalonate attenuated the up-regulation of RhoB levels by simvastatin (Fig. 3*A*, *right*). To ascertain whether this effect was dependent on some specific products of mevalonate we explored the ability of various isoprenoid intermediates to circumvent simvastatin-induced up-regulation of RhoB (Fig. 3*A*, *right*). Incubation of BAEC with farnesyl-pyrophosphate during treatment with simvastatin slightly reduced the up-regulation of RhoB protein levels whereas geranylgeranyl-pyrophosphate (GGPP) had a protective effect similar to that of mevalonate. This effect of GGPP was not restricted to BAEC because it was also observed in HeLa and NIH-3T3 cells (results not shown).

The RhoB protein and transcript are short-lived species, the levels of which can be modulated both transcriptionally and posttranscriptionally (26, 29). To get insight into the mechanism of simvastatin-induced RhoB up-regulation we explored the levels of RhoB mRNA. As it can be observed in Fig. 3*B* simvastatin augmented the levels of RhoB transcript by ~2-fold, an extent lower than that observed at the protein level. The effect of simvastatin was specific for RhoB as evidenced by the observation that the levels of β-actin did not increase or were slightly decreased by simvastatin treatment. Cholesterol supplementation did not increase RhoB mRNA levels appreciably and was also ineffective at preventing the effect of simvastatin. RhoB mRNA levels followed a pattern similar to that observed for the RhoB protein in response to supplementation with mevalonate, farnesyl-pyrophosphate, and GGPP (Fig. 3*B*, *right*). The ability of GGPP to prevent simvastatin-induced RhoB up-regulation suggested the involvement of geranylgeranylated proteins in this effect. Because most G-proteins are modified by geranylgeranylation, we took advantage of the availability of bacterial toxins that selectively inactivate specific types of G-proteins to assess their effect on RhoB levels (Fig. 4). Treatment of BAEC with pertussis toxin, which inhib-

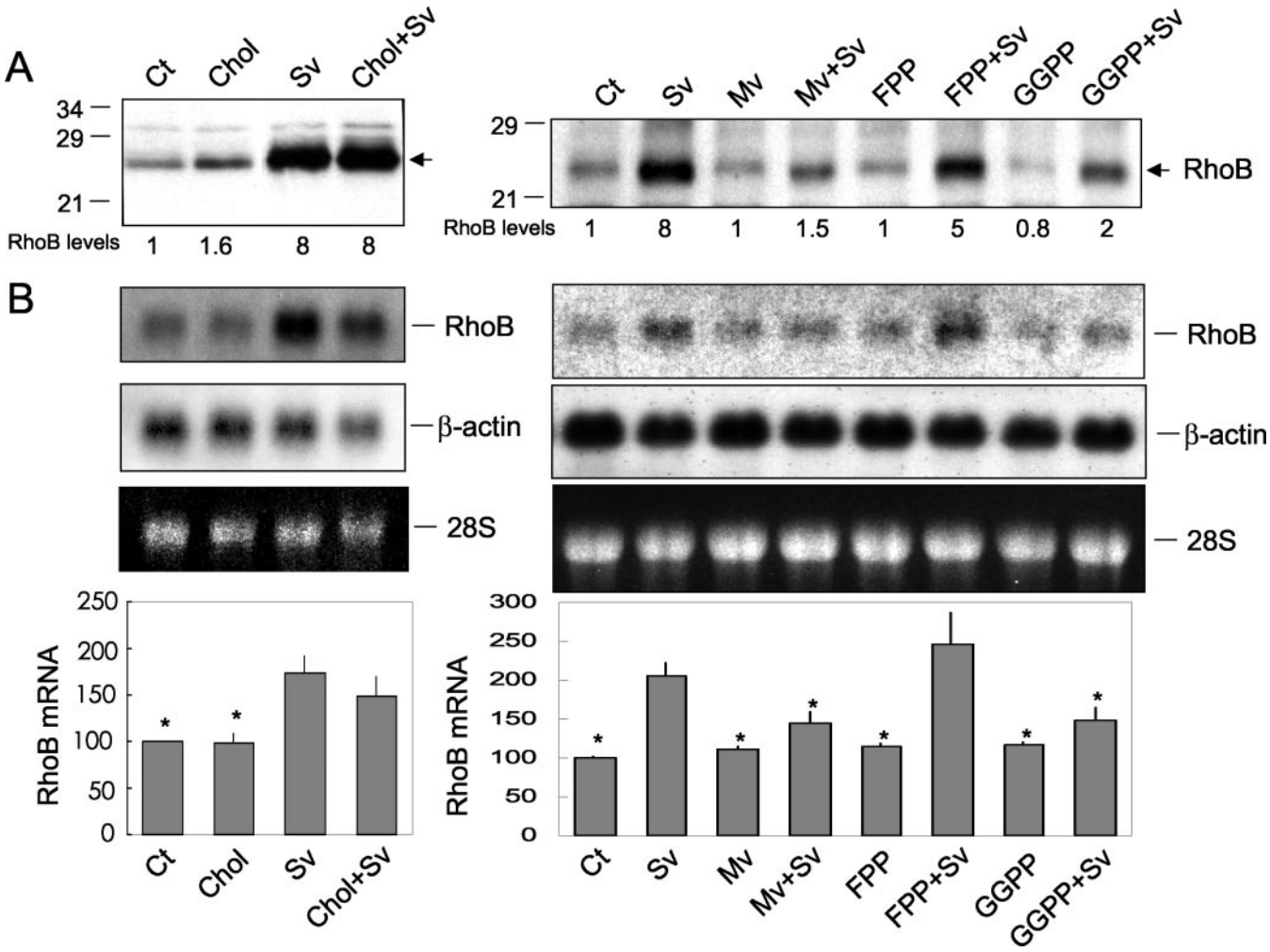


FIG. 3. **Effect of cholesterol availability and isoprenoid intermediates on RhoB protein and mRNA abundance.** *A*, BAEC were incubated with the indicated agents for 24 h and RhoB protein levels were estimated by immunoblot. *Sv*, 10 μM simvastatin; *Chol*, 100 μg/ml cholesterol; *Mv*, 100 μM mevalonic acid; *FPP*, 5 μM farnesyl-pyrophosphate; *GGPP*, 5 μM geranylgeranyl-pyrophosphate. Levels of RhoB protein estimated by image scanning of ECL exposures, expressed in arbitrary units, are shown at the bottom. Results are representative of at least three experiments. *B*, BAEC were treated with the indicated agents as above. RhoB mRNA levels were determined by Northern blot. Blots were hybridized first with RhoB cDNA and reprobed with β-actin cDNA. *Lower panels* depict the densitometric analysis of RhoB mRNA levels after correction by the expression of β-actin. Data are mean ± S.E. of three independent experiments. *, $p < 0.05$ *versus* simvastatin by Student's *t* test.

its G_i proteins, or with cholera toxin, which stimulates G_s proteins did not alter RhoB protein levels significantly (Fig. 4*A*). In contrast, treatment with either toxin B from *Clostridium difficile*, or with *Clostridium botulinum* C3 exoenzyme, known to block the activity of Rho proteins, increased RhoB levels, although to a lesser extent than simvastatin (Fig. 4*B*). Moreover, the Rho kinase inhibitor Y-27632 also augmented RhoB protein levels moderately. Interestingly, both toxin B and Y-27632 induced a similar increase in RhoB mRNA levels (Fig. 4*C*). Taken together, these observations suggest that the increase in RhoB mRNA expression induced by simvastatin could be mediated by the impairment geranylgeranylated proteins, among which Rho proteins are good candidates. However, the higher extent of amplification of RhoB protein levels by simvastatin suggests that there may be translational or posttranslational mechanisms that contribute to this effect.

Effect of Simvastatin on RhoB Protein Stability—RhoB protein degradation is an important process for the control of RhoB protein levels (26). We then explored whether this process was affected by simvastatin. In control cells, inhibition of protein synthesis with cycloheximide lead to a rapid decrease in RhoB protein levels, with less than 50% RhoB protein remaining after 6 h (Fig. 5*A*). In contrast, in cells pre-treated with simvastatin for 24 h, the decay of RhoB protein levels was significantly reduced, thus suggesting the contribution of posttranslational events to simvastatin-elicited RhoB up-regulation. Treatment with simvastatin at the time of cycloheximide addition did not result in increased RhoB levels (Fig. 5*B*). Also, in these co-treatment experiments, the decay of RhoB levels was not reduced, even when a concentration of 40 μM simvastatin was used ($n = 2$, results not shown), thus making unlikely the possibility of a direct interference of simvastatin with the activity of cellular proteases.

Effect of Simvastatin on RhoB Subcellular Distribution and Processing—Decreased degradation of RhoB in simvastatin-treated cells could result from alterations in RhoB subcellular localization and/or function due to impaired posttranslational processing. To test this hypothesis we assessed the effect of simvastatin on RhoB subcellular distribution and processing. In confluent, serum-deprived BAEC, RhoB was predominantly membrane-bound (Fig. 6*A*). In simvastatin-treated cells, the amount of membrane-bound RhoB was significantly reduced, whereas the increase in RhoB protein was exclusively localized to the soluble fraction. Treatment with simvastatin also led to a decrease in the electrophoretic mobility of RhoB protein, which is indicative of the lack of posttranslational modification

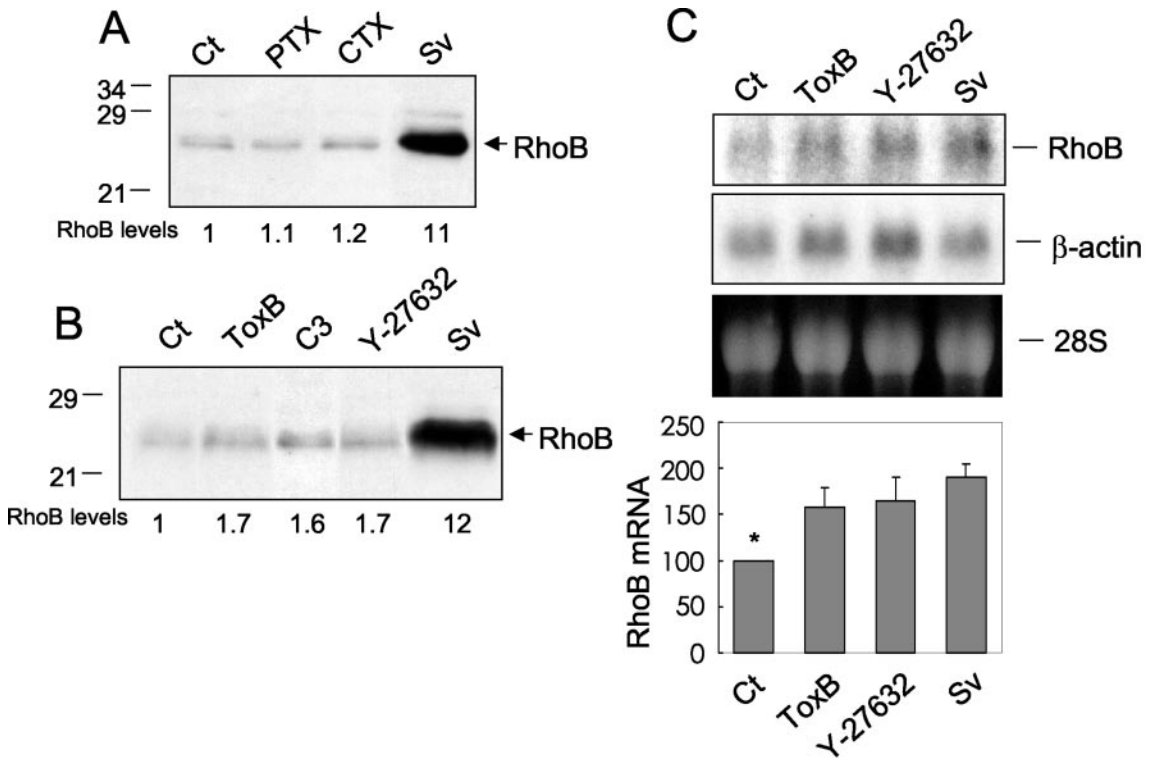


FIG. 4. **Effect of bacterial toxins on RhoB levels.** BAEC were treated with the indicated agents for 24 h, and the levels of RhoB protein and mRNA were assessed by Western (*A and B*) or Northern blot (*C*). *PTX*, 0.5 μg/ml pertussis toxin; *CTX*, 0.5 μg/ml cholera toxin; *ToxB*, 0.5 ng/ml toxin B from *Clostridium difficile*; *C3*, 50 μg/ml *Clostridium botulinum* C3 exoenzyme; *Y-27632*, 5 μM Rho-associated kinase inhibitor, *Sv*, 10 μM simvastatin Results shown are representative of at least three experiments. Levels of RhoB protein and mRNA estimated as in Fig. 3 are expressed in arbitrary units. *, $p < 0.05$ *versus* simvastatin by Student's *t* test.

(26) (Fig. 6*B*). Thus, in simvastatin-treated cells, most of RhoB protein is accumulated in the cytosol in its non-isoprenylated form.

RhoB Isoprenylation Is Necessary for Degradation—To ascertain whether the effect of simvastatin was related to the inhibition of protein isoprenylation we first explored the ability of the inhibitors of protein farnesyltransferase and geranylgeranyltransferase I, FTI-277 and GGTI-286, respectively, to increase RhoB levels (Fig. 7*A*). We observed that FTI-277 slightly increased the levels of RhoB protein and GGTI-286 induced a 3.8-fold increase of RhoB expression in BAEC ($n = 4$). The combination of the two inhibitors induced a potent increase in RhoB protein levels, that was comparable with that attained with simvastatin (8-fold increase, average of four experiments). A similar effect of prenyltransferase inhibitors was observed in NIH-3T3 fibroblasts (Fig 7*A*). We next evaluated whether non-isoprenylated, cytosolic RhoB displays increased stability with respect to the wild-type protein. For this purpose, we generated wild-type and isoprenylation-deficient RhoB constructs and assessed their subcellular localization and stability in transient transfection experiments (Fig. 7*B*). GFP-HA-RhoBwt showed a preferential perinuclear distribution when transfected in NIH-3T3 fibroblasts, with a particulate pattern indicative of membrane association. In contrast, GFP-HA-RhoB-C193S showed a diffuse distribution, indistinguishable from that of GFP (data not shown), typical of soluble proteins. Treatment of GFP-HA-RhoBwt-transfected fibroblasts with simvastatin caused a redistribution of RhoB from the membrane-associated to the soluble compartment. We next explored the effect of simvastatin on the expression of transiently transfected wild-type and mutant RhoB proteins by Western blot and detection with an anti-HA antibody (Fig. 7*C*). Simvastatin potently increased the levels of HA-RhoBwt, suggesting that its effect largely depends on the RhoB coding sequence. The levels of HA-RhoB-C193S expressed in cells were consistently higher than those of HA-RhoBwt and were not further increased by simvastatin, suggesting that inhibition of RhoB isoprenylation is required for simvastatin effect. Finally, we estimated the half-life of both proteins. As shown in Fig. 7*D*, HA-RhoBwt levels decayed rapidly upon inhibition of protein synthesis, displaying a stability similar to that of endogenous RhoB (Fig. 5). In contrast, HA-RhoB-C193S was markedly more stable. These results show that RhoB isoprenylation is required for rapid turnover of this protein.

DISCUSSION

The results presented in this work show that RhoB levels are modulated by the cholesterol biosynthetic pathway. Down-regulation of this pathway either by means of HMG-CoA reductase inhibitors or excess cholesterol is associated with an increase in the amount of RhoB. This regulation occurs in part at the mRNA level and largely at the protein level, because inhibition of RhoB isoprenylation either by pharmacological or genetic manipulation markedly reduces the degradation of this protein.

The cholesterol biosynthetic pathway provides the isoprenoid intermediates needed for the posttranslational modification of G-proteins. Although protein isoprenylation is an irreversible modification, it is susceptible to a certain degree of regulation, because the activity of the prenyltransferases may be controlled by some growth factors and by the availability of isoprenoids. The cholesterol pathway is subjected to a complex feedback regulation (1), and it can be modulated by stress (39). In recent years it has become evident that the activity of this pathway may control the maturation of certain G-proteins such as Ras, thus resulting in the regulation of Ras-mediated cellu-

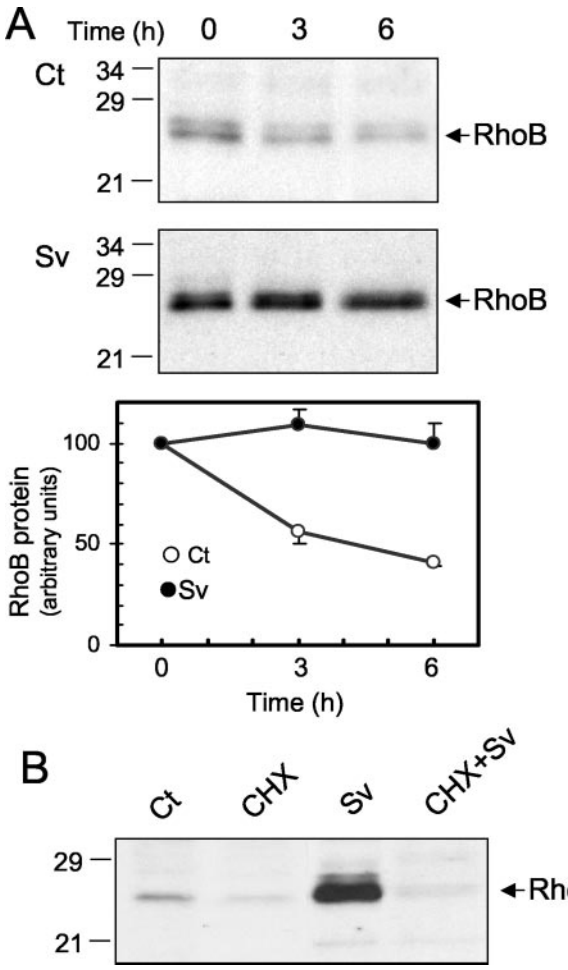


FIG. 5. **Effect of simvastatin on RhoB protein degradation.** *A*, BAEC were treated in the absence (*Ct*) or presence of 10 μM simvastatin (*Sv*), as indicated. After 24 h, protein synthesis was blocked by addition of 20 μg/ml cycloheximide, and RhoB protein levels were estimated by immunoblot at several time points after cycloheximide addition. ECL exposures shown correspond to a 4-min exposure for *Ct* and a 30-s exposure for *Sv*. Results shown in the *lower panel* are average values ± S.E. of three experiments. *B*, BAEC were treated in the absence (*Ct*) or presence of 10 μM simvastatin and/or 20 μg/ml cycloheximide (*CHX*) for 24 h, and levels of RhoB were assessed by immunoblot.

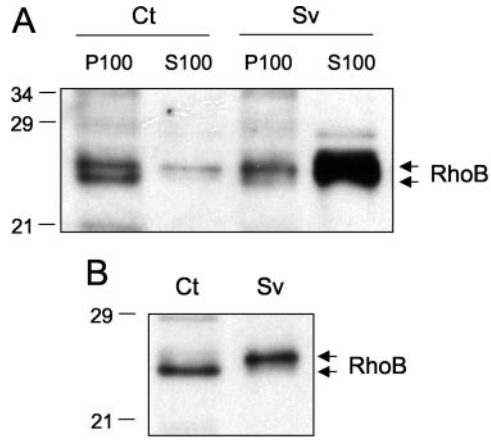


FIG. 6. **Effect of simvastatin on RhoB subcellular localization and processing.** BAEC were incubated in the absence or presence of 10 μM simvastatin for 24 h. *A*, S100 (cytosol) and P100 (membrane) fractions were obtained and 10 μg from each fraction were analyzed by immunoblot. *B*, electrophoretic mobility of RhoB protein present in cell lysates from control (20 μg of total protein/lane) or simvastatin-treated cells (4 μg/lane) was assessed by immunoblot. A lower amount of protein was analyzed in the case of simvastatin-treated cells to avoid the lack of resolution caused by RhoB overexpression. Results are representative of at least three experiments.

lar processes in response to cholesterol levels or cellular stress (39, 40). In addition, several lines of evidence indicate that the mevalonate pathway may influence the expression of other members of the Ras superfamily, namely Ras1 and Ras2 in yeast (23), and Rab5 and Rab7 in thyroid cells (24), through mechanisms not yet completely elucidated.

The relationship between G-protein expression and cholesterol metabolism may be bidirectional. In a recent report, cdc42 proteins have been involved in the regulation of cholesterol efflux (41). In addition, in fibroblasts from patients with the inherited disorder of lipid metabolism known as Tangier disease, characterized by cellular accumulation of cholesteryl esters, the expression of several Rho proteins, including RhoB, is increased (42). On this basis, a role for Rho proteins in cholesterol transport has been hypothesized. Our results raise the possibility that the observed increase in Rho proteins expression may be caused in part by the accumulation of cholesterol in cells.

The mechanisms by which the mevalonate pathway regulates RhoB appear to be multiple. Recently, mevalonate depletion has been reported to result in an increase in RhoB mRNA (25). However, the involvement of isoprenoid intermediates or isoprenylated proteins in this effect has not been explored. Here we have shown that inhibition of mevalonate biosynthesis with simvastatin moderately up-regulates RhoB mRNA levels and that this effect is prevented by GGPP. In addition, we have observed that inhibition of Rho signaling by means of bacterial toxins that target and inactivate Rho proteins (43) or by a Rho kinase inhibitor, is also associated with a moderate increase in RhoB protein and mRNA levels. These observations suggest that geranylgeranylated proteins and, in particular, Rho proteins, exert a negative feedback on RhoB mRNA expression in endothelial cells. This interpretation is in accordance with previous studies that identified a negative role of RhoA and B on the activity of the mouse RhoB promoter (29). Our results indicate that the main effect of simvastatin on RhoB expression takes place at a posttranslational level by reducing RhoB degradation. This could occur through several mechanisms. Simvastatin could affect the activity of proteolytic pathways within the cell. In fact, statins have been reported to modulate the activity of the proteasome (44, 45), a protein-degradation pathway proposed to mediate RhoB turnover (26). However, the effect of simvastatin on RhoB levels and stability was completely prevented by co-treatment with cycloheximide, thus showing that *de novo* protein synthesis is required for its effect. Moreover, inhibition of protein isoprenylation by using peptidomimetic inhibitors of prenyltransferases reproduced the effect of simvastatin on RhoB levels. These observations suggest that the effect of simvastatin is related to the inhibition of protein isoprenylation rather than to a direct interference with cellular proteases. Because the increase in RhoB protein elicited by simvastatin occurs at the expense of non-prenylated cytosol-partitioned RhoB, it could be hypothesized that the immature cytosolic protein is a poorer substrate for proteases or it does not localize to the appropriate cellular compartment for degradation. This is supported by the fact that the Cys193Ser RhoB mutant, which lacks the isoprenylation site and is cytosolic, is markedly more stable than the wild-type protein. These observations indicate that isoprenylation of RhoB determines the half-life of the protein. Interestingly, carboxyl methylation of the isoprenylated cysteine in RhoA has been reported to reduce its turnover (46), thus providing another example of the regu-

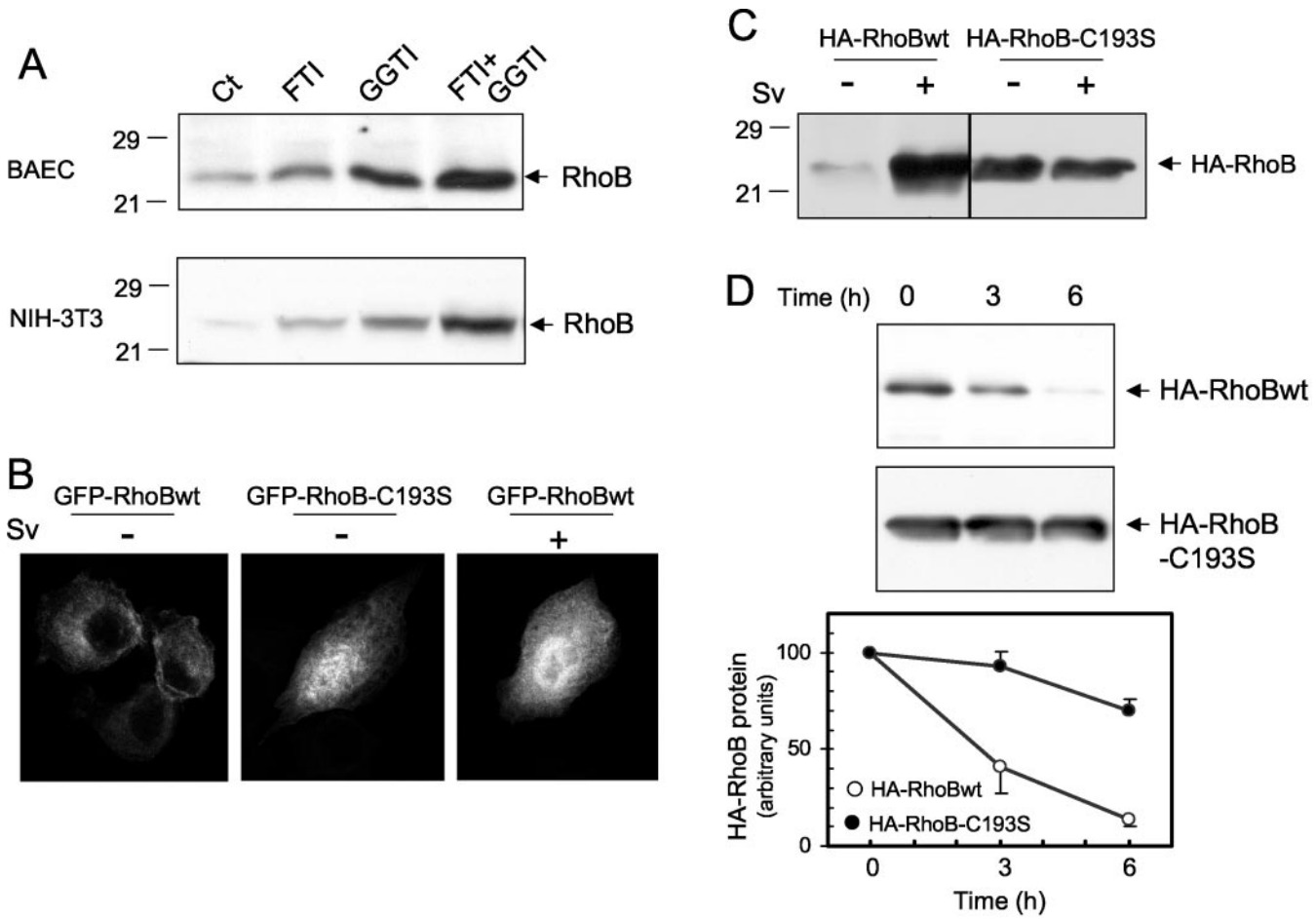


FIG. 7. **Importance of isoprenylation for RhoB protein stability.** *A*, BAEC or NIH-3T3 fibroblasts, as indicated, were treated with the farnesyltransferase inhibitor FTI-277 and/or the geranylgeranyltransferase I inhibitor GGTI-286 at 5 μM for 24 h. Levels of RhoB protein were visualized by immunoblot. *B*, NIH-3T3 fibroblasts transfected with the indicated GFP-RhoB constructs were treated in the absence or presence of 10 μM simvastatin for 24 h, and the subcellular localization of GFP-RhoB was visualized by confocal fluorescence microscopy. *C*, NIH-3T3 fibroblasts transfected with wild type (*wt*) or isoprenylation-deficient (*C193S*) HA-RhoB expression plasmids were plated on 6-well dishes and cultured in the absence or presence of 10 μM simvastatin for 24 h, as indicated. Levels of exogenous wild-type and C193S RhoB proteins were estimated by Western blot with an anti-HA antibody. *D*, decay of wild-type and C193S HA-RhoB levels at the indicated time points after addition of cycloheximide was estimated as above. ECL exposures shown correspond to a 2-min exposure for wild-type and a 20-s exposure for C193S HA-RhoB. Results shown in the *lower panel* are average values ± S.E. of four experiments.

lation of Rho protein stability as a function of their posttranslational processing.

Whether the increased stability of unprenylated RhoB is caused by changes in subcellular distribution, activity, or ability to interact with other cellular proteins will be the subject of further studies. Although inhibition of RhoB isoprenylation may abolish some of RhoB functions, like its cell transforming ability (47) or the activation of the endothelin-1 promoter (10), other functions could be conserved, as it has been described for the activation of the serum-response element-dependent transcription in overexpression experiments (47). Future work will address whether unprenylated RhoB may have specific functions or whether it behaves as a dominant-negative RhoB protein.

In conclusion, we have found that RhoB mRNA levels and protein stability can be regulated by the availability of isoprenoid intermediates needed for protein modification. These findings unveil the novel role of isoprenylation in the control of RhoB protein degradation and may contribute to the understanding of the complex structure-function relationships of RhoB posttranslational modifications.

Acknowledgments—We thank Dr. S. Lamas for helpful comments and discussion, Dr. M. Díaz-Cazorla for culture of human umbilical vein endothelial cells and E. Soria and M. J. Carrasco for technical assistance.

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