Résultats : Partie II

Régulation négative de RhoB par ses homologues RhoA et RhoC via RhoGDIα

Introduction

Comme nous l'avons décrit dans l'introduction générale, la famille des RhoGTPases se divise en huit sous-groupes. Parmi ceux-ci, on distingue la sous-famille Rho comprenant RhoA, son homologue RhoC mais également RhoB. Contrairement à RhoA et RhoC, RhoB est une protéine dont la demi-vie est courte et qui possède des propriétés anti-tumorales. En effet, certaines tumeurs présentent une diminution d'expression de RhoB tandis que sa surexpression inhibe la migration cellulaire, la croissance tumorale et la formation de métastases. Par ailleurs, il a été démontré que RhoB fait partie de la réponse antitumorale induite par des agents anticancéreux tels que les inhibiteurs de la farnésyltransférase. La compréhension des mécanismes régulant son expression pourrait ouvrir de nouvelles voies de développement de stratégies anticancéreuses.

Résumé des résultats

- Dans le premier chapitre de notre mémoire, nous avons montré que l'inhibition spécifique de RhoA ou de RhoC seuls ne modifiait pas l'organisation et la distribution de l'actine polymérique dans les

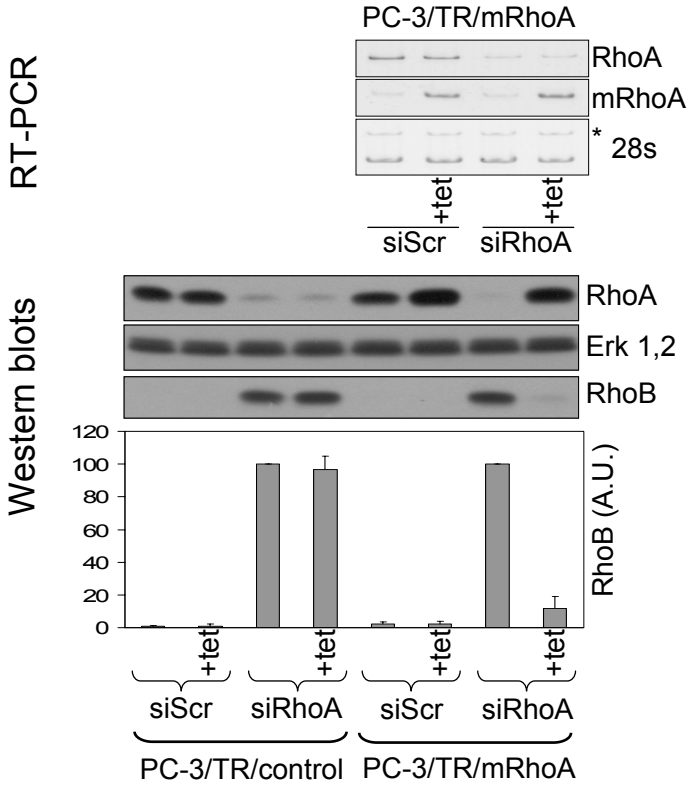

Figure 14: Réversion de la régulation de RhoB par réexpression de RhoA. *Les clones inductibles contenant le vecteur vide (PC-3/TR/control) ou le vecteur permettant l'expression de l'ARNm de RhoA muté de façon à le rendre résistant au siRNA (PC-3/TR/mRhoA) mais exprimant toujours la protéine sauvage sont transfectés par siScr ou siRhoA en présence (+tet) ou en l'absence de tétracycline.* ***Panneau supérieur.*** *RT-PCR réalisée avec des oligonucléotides permettant uniquement l'amplification de l'ARNm RhoA endogène ou uniquement l'amplification de l'ARNm RhoA exogène résistant au siRNA (mRhoA).* ***Panneaux inférieurs.*** *Western blot sur des lysats cellulaires totaux et quantification de RhoB après normalisation sur base de l'abondance de Erk 1,2.*

fibroblastes humains. Puisque ces deux RhoGTPases activent un effecteur commun (ROCK) qui contrôle la formation des fibres de stress d'actomyosine en permettant la phosphorylation de la chaîne légère de la myosine, nous avons réalisé une double transfection par siRhoA et siRhoC et évalué les fibres de stress par marquage à la phalloïdine dans des cellules d'adénocarcinome mammaire (Hs578T). Malgré une extinction efficace de l'expression de ces deux RhoGTPases et une altération modérée de la morphologie, les fibres de stress étaient toujours présentes ce qui suggérait l'existence d'une activité de type Rho résiduelle. L'inhibition de RhoA seul et la double répression de RhoA et de RhoC induisent effectivement l'apparition d'un taux élevé de la protéine RhoB dans les Hs578T. Cette surexpression s'est également révélée dans d'autres types cellulaires tels que les fibroblastes dermiques primaires, des cellules de mélanome, de fibrosarcome et d'adénocarcinome prostatique (PC-3). Ce n'est que lorsque les Hs578T sont transfectés simultanément par les siRNAs ciblant RhoA, RhoC et RhoB que la disparition totale des fibres de stress est observée. Cette observation indique que RhoB induit par l'extinction simultanée de RhoA et RhoC joue un rôle actif dans la dynamique du cytosquelette. Ceci est corroboré par la mesure de la forme active de RhoB par « Pull-down » indiquant que la forme RhoB-GTP est accrue parallèlement à l'induction de la protéine. La réexpression de RhoA à l'aide d'un vecteur inductible exprimant un ARNm muté insensible au siRNA a permis la réversion de l'induction de RhoB confirmant ainsi l'existence d'un processus de régulation de RhoB par RhoA (Figure 14).

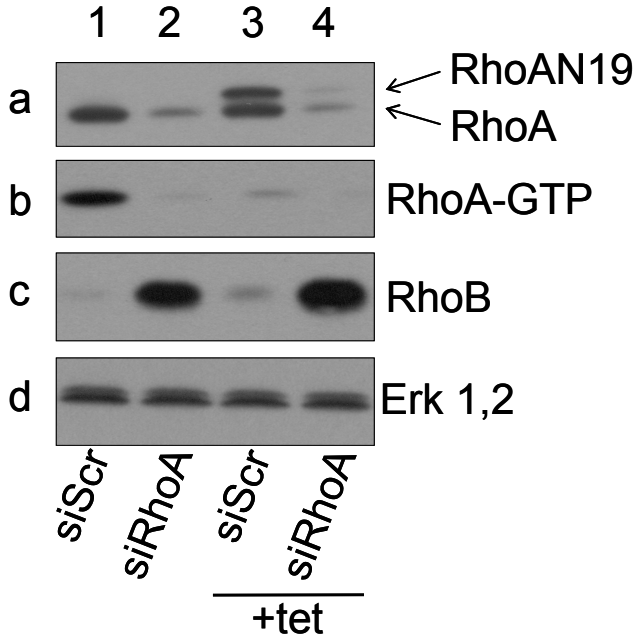

Figure 15 *:* ***RhoB est régulé par RhoA-GDP****. Les clones PC-3 inductibles contenant le vecteur permettant l'expression de RhoA dominant négatif (PC-3/TR/RhoA N19) sont transfectés par siScr ou par siRhoA en présence (+tet) ou en l'absence de tétracycline. Western blot utilisant les anticorps anti-RhoA, anti-RhoB et anti-Erk 1,2 sur les lysats totaux (a, c, d) ou après pull-down de RhoA-GTP (b).*

- L'expression inductible de la forme dominante négative de RhoA (RhoAN19) empêche l'échange GDP-GTP (Figure 15, a et b, puits 3) et démontre que l'absence de la forme GTP n'est pas responsable de l'induction de RhoB (Figure 15, c, puits 3) suggérant que c'est la suppression de la forme RhoA-GDP qui en est responsable (Figure 15, c, puits 4).

- La surexpression de RhoB est liée à un allongement de la demi-vie de la protéine. Cette stabilisation induite par l'ablation de RhoA est indépendante des voies de synthèse des isoprénoïdes, de ROCK et des voies de signalisation de Erk 1,2, p38 MAPK ou PI3K mais elle implique en revanche l'inhibiteur de dissociation RhoGDIα. En effet, la surexpression de RhoGDIα accroît la demi-vie de RhoB tandis que la co-transfection d'un siRhoGDIα avec le siRhoA réduit l'induction de RhoB (Figure 16, comparer les puits 3 et 4 aux puits 6 et7). Par ailleurs, l'expression d'un mutant de RhoA ne se liant pas à RhoGDIα ne permet pas la réversion de l'induction de RhoB suite à l'extinction de RhoA.

Conclusions

Nos résultats suggèrent que l'inhibition de l'expression d'une RhoGTPase ne mime pas uniquement les effets de la surexpression d'un mutant dominant-négatif mais révèle de nouvelles voies de régulation des RhoGTPases. Ceci est dû non seulement à la haute spécificité des siRNAs mais également à la déplétion simultanée des formes liées au GTP et au GDP ce qui nous a permis de mettre en évidence une fonction inattendue de RhoA-GDP. Les mécanismes de

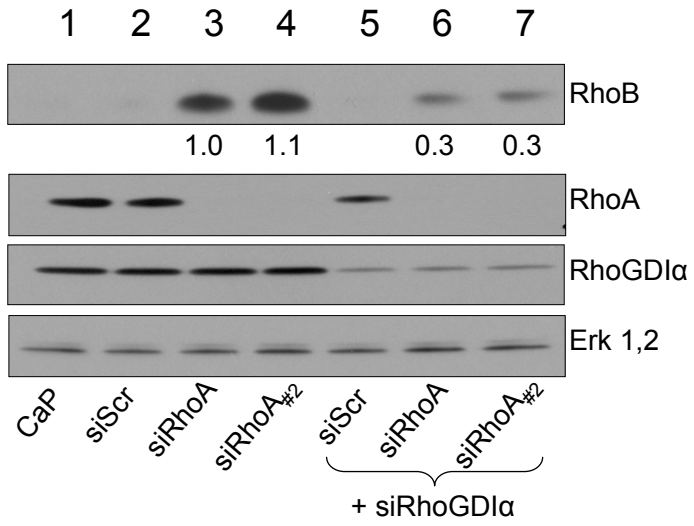

Figure 16: L'induction de RhoB est dépendante de RhoGDIα*. Les HS578T sont transfectées par l'agent de transfection seul (CaP), siScr, siRhoA, siRhoA#2, siScr+siRhoGDIα, siRhoA+siRhoGDIα et siRhoA#2+siRhoGDIα. Western blot réalisé sur des extraits cellulaires totaux utilisant des anticorps anti-RhoB, anti-RhoA, anti-RhoGDIα et anti-Erk1,2.*

régulation croisée ne se limitent probablement pas à la seule sous-famille Rho et doivent être pris en compte pour l'analyse des résultats d'expériences basées sur le ciblage des RhoGTPases par des siRNA mais également pour l'élaboration de stratégies à visée thérapeutique.

Supplemental Material can be found at:
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RhoA-GDP Regulates RhoB Protein Stability

POTENTIAL INVOLVEMENT OF RhoGDIα[*S]

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RhoA plays a significant role in actin stress fibers formation. However, silencing RhoA alone or RhoA and RhoC did not completely suppress the stress fibers suggesting a residual "Rho-like" activity. RhoB, the third member of the Rho subclass, is a short-lived protein barely detectable in basal conditions. In various cell types, the silencing of RhoA induced a strong up-regulation of both total and active RhoB protein levels that were rescued by re-expressing RhoA and related to an enhanced half-life of the protein. The RhoA-dependent regulation of RhoB does not depend on the activity of RhoA but is mediated by its GDP-bound form. The stabilization of RhoB was not dependent on isoprenoid biosynthesis, Rho kinase, extracellular signal-regulated kinase, p38 mitogen-activated kinase, or phosphatidylinositol 3′-OH kinase pathways but required RhoGDIα. The forced expression of RhoGDIα increased RhoB half-life, whereas its knock-down antagonized the induction of RhoB following RhoA silencing. Moreover, a RhoA mutant (RhoAR68E) unable to bind RhoGDIα was significantly less efficient as compared with wild-type RhoA in reversing RhoB up-regulation upon RhoA silencing. These results suggest that, in basal conditions, RhoGDIα is rate-limiting and the suppression of RhoA makes it available to stabilize RhoB. Our results highlight RhoGDIα-dependent cross-talks that regulate the stability of RhoGTPases.

The small GTPases of the Rho family are at the cross-roads of signaling pathways initiated by receptors to diffusible biological mediators and those depending on cell-adhesion receptors. They are key signaling molecules regulating a plethora of biological pathways (1). The Rho GTPases shuttle between an inactive GDP-bound state and an active GTP-bound state. Their level of activation is regulated by three classes of factors: the guanine-nucleotide exchange factors that catalyze the exchange of GDP to GTP, the GAPs that increase the intrinsic GTPase activity of the RhoGTPase, and the RhoGDIs that inhibit the exchange of GDP to GTP. However, their mechanism of action may not be solely restricted to activation of downstream signaling cascades when GTP-loaded (2).

In mammals, RhoGDIs constitute a family encompassing three members: RhoGDIα, the ubiquitously expressed archetypal member of the family; Ly/D4-GDI or RhoGDIβ, which has a hematopoietic tissue-specific expression pattern; and RhoGDI-3 or -γ, which is membrane-anchored and preferentially expressed in brain, pancreas, lung, kidney, and testis. RhoGDIs are usually perceived as "static" inhibitors preventing the activation of the downstream effectors by the RhoGTPases. Accumulative evidences suggest that RhoGTPase-RhoGDI complexes are highly dynamic. Phosphorylation of RhoGDIα by various kinases that decreases its affinity for RhoGTPases is one mechanism used by receptors to activate specific RhoGTPases (3, 4). By contrast, phosphorylation of the RhoGTPases themselves seems to increase their affinity for RhoGDIα thus leading to signal termination (5–7). Furthermore, a novel function has been attributed to RhoGDIα in the activation of NADPH oxidase by its capacity to present the RhoGTPase to the appropriate effectors in a way that potentiates efficient activation (8, 9). More recently, it was shown that a Rac1 mutant displaying an increased affinity for RhoGDIα stimulated the activity of RhoA suggesting that RhoGDI is a key intermediate in the cross-talks between RhoA and Rac1 (10).

Among the RhoGTPase family, the Rho subclass includes RhoA, its closely related homolog RhoC, and RhoB. By contrast to RhoA and RhoC, RhoB is a short-lived protein displaying anti-tumorigenic properties (11). In various solid tumors, RhoB expression diminished in parallel with tumor progression (12, 13). Its overexpression antagonizes cell migration, tumor growth, and metastasis (14, 15). Furthermore, RhoB was reported to be an essential component of the anti-tumoral response triggered by farnesyl transferase inhibitors as well as of the apoptotic response of transformed cells to DNA damaging agents (16, 17). Thus, a better understanding of the mechanisms regulating RhoB expression and stability is of great importance for the optimization of potential anti-cancer strategies.

In this report, we targeted RhoGTPases by using a siRNA[4]-based approach. This technology that recently allowed us to

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[4] The abbreviations used are: siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; HSF, human skin fibroblast; p38MAPK, p38 mitogen-activated kinase; PI3K, phosphatidylinositol 3′-OH kinase; ROCK, Rho kinase; RT, reverse transcriptase; HA, hemagglutinin; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

highlight the regulation operated by Cdc42 on matrix metalloprotease-1 (18) has several advantages as compared with classical methods. Beside a higher specificity, siRNA suppresses both GDP- and GTP-bound forms allowing to better evaluate the contribution of either form in the global function of individual RhoGTPase. Silencing RhoA led to an increased RhoB expression by a post-transcriptional mechanism extending the half-life of the protein. Our results demonstrated that this regulation is mediated by RhoA-GDP. Investigation of various intracellular signaling pathways suggested a role of a RhoGDIα-dependent mechanism in this process.

EXPERIMENTAL PROCEDURES

Reagents and Cells—The antibodies were purchased from the following manufacturers: mouse anti-RhoA (sc-418), rabbit anti-RhoB (number sc-180), goat anti-RhoC (number sc-26480), rabbit anti-RhoGDIα (number sc-360), and normal rabbit IgG (number sc-2027) were from Santa Cruz Biotechnology; mouse anti-Rac1 (23A8) from Upstate Biotechnology; mouse anti-Cdc42 from BD Biosciences; rabbit anti-ERK1,2 (number M-5670) was from Sigma; rabbit monoclonal anti-β-actin (13E5, number 4970), rabbit polyclonal anti-AKT (number 9272), anti-phospho-AKT (Ser-473, number 9271), and anti-phospho ERK1,2 (number 4370X) were from Cell Signaling; mouse monoclonal anti-human p38 (AHO0782) was from BioSource International; rabbit anti-phospho-p38 was from Zymed Laboratories Inc. (number 36-8500); rabbit polyclonal anti-Tet-repressor antibody was from MoBiTec (number TET01); rat anti-HA (clone 3F10, number 11867423001) was from Roche Diagnostics. Alexa Fluor® 546 goat anti-mouse IgG (number A11003) and Alexa Fluor® 488 goat anti-rabbit IgG were from Molecular Probes. Y-27632 was kindly supplied by A. Yoshimura (Welfide Co., Japan), U0126 was from Calbiochem, LY294 from Sigma, and SB203580 from Alexis Corporation. 4′,6-Diamidino-2-phenylindole (Bisbenzimide H 33258 number 382061) was from Calbiochem. Blasticidin (number R210-01) and Zeocin™ (number 45-0430) were from Invitrogen. Human primary skin fibroblasts (HSF) were isolated by the explant procedure from normal human dermis and cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Cambrex). Cells were trypsinized 1:3 every week and used between passages 8 and 13. Human breast adenocarcinoma cell line HS578T, human fibrosarcoma cell line HT1080, and human melanoma cell line A2058 were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum and trypsinized 1:10 every week. Human prostate adenocarcinoma cells PC-3 were amplified in F-12 Kaighn's medium supplemented with 7% fetal bovine serum.

siRNA Transfection—A series of 21-nucleotide long siRNAs were chemically synthesized, desalted, deprotected, and PAGE purified (Eurogentec). The 5′-GAAGUCAAGCAUUUCUGUCTT-3′ and 5′-GACAGAAAUGCUUGACUUCTT-3′ oligoribonucleotides (siRhoA) or 5′-GAAGGAUCUUCGGAAUGAUTT-3′ and 5′-AUCAUUCCGAAGAUCCUUCTT-3′ oligoribonucleotides (siRhoA#2) were used to inhibit RhoA synthesis; the 5′-GAAGGACCUGAGGCAAGACTT-3′ and 5′-GUCUUGCCUCAGGUCCUUCTT-3′ oligoribonucleotides (siRhoC) or the 5′-UCCACUUCUCAGGAAUGUUTT-3′ and 5′-AACAUUCCUGAGAAGUGGATT-3′ oligoribonucleotides (siRhoC#2) were used to inhibit RhoC synthesis; the 5′-CACCACUGUCCCAACACUCTT-3′ and 5′-GAGUGUUGGGACAGUGGUGTT-3′ oligoribonucleotides (siRac1) or 5′-GAUAAAGACACGAUCGAGATT-3′ and 5′-UCUCGAUCGUGUCUUUAUCTT-3′ oligoribonucleotides (siRac1#2) were used to inhibit Rac1 synthesis. The siRNAs used to inhibit RhoB, Cdc42, or RhoGDIα synthesis were described elsewhere (18–20). The 5′-UUGCAUACAGGACUCGUUATT-3′ and 5′-UAACGAGUCCUGUAUGCAATT-3′ oligoribonucleotides used as control (siScr) were designed by randomly mixing the sequence of the siRhoA. The 5′-AAAGUCAAUCAUUUCUGUCTT-3′ and 5′-GACAGAAAUGAUUGACUUUTT-3′ (CtrRhoA) and 5′-AAAGGACCCGAGGCAAGACTT-3′ and 5′-GUCUUGCCUCGGGUCCUUUTT-3′ (CtrRhoC) were used, respectively, as specific controls for siRhoA and siRhoC. They were designed by introducing two nucleotide mismatches in the sequences of siRhoA and siRhoC, respectively. Each pair of oligoribonucleotides was annealed at a concentration of 20 μM in 50 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. siRNA transfection was carried out as previously described (21). Briefly, calcium phosphate-mediated transfection was performed overnight (14–16 h) on subconfluent cells at a final concentration of siRNA ranging from 0.2 to 60 nM. Cells were washed twice with PBS and once with complete medium, this last step was defined as time 0 post-transfection. Cells were lysed for Western blot or RT-PCR analysis 48 h post-transfection.

RT-PCR Analysis—The RT-PCR amplifications were performed in an automated thermocycler (GeneAmp PCR system 9600) using a GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (PerkinElmer Life Sciences) with pairs of primers amplifying mRNA coding for human RhoA (5′-GTACATGGAGTGTTCAGCAAAGACC-3′ and 5′-GGTGGGCCAGACGGGTTGGACA-3′), human mutated RhoA (mRhoA) (5′-GTACATGGAGTGTTCAGCAAAGACC-3′ and 5′-AGAAGGCACAGTCGAGGCTGATCA-3′), and 28 S rRNA (5′-GTTCACCCACTAATAGGGAACGTGA-3′ and 5′-GGATTCTGACTTAGAGGCGTTCAGT-3′). For the 28 S rRNA, the efficiency of the RT-PCR was controlled by a synthetic RNA co-transcribed and co-amplified with the same primers as the endogenous RNA to yield an amplification product of slightly larger size. The RT-PCR conditions were described elsewhere (18). Briefly, 10 ng of total RNA and a known copy number of the standard synthetic RNA were reverse transcribed (70 °C for 15 min). Then, RNA-DNA heteroduplexes were denatured for 2 min at 94 °C and amplification was carried out for 22 cycles (RhoA and mRhoA) or for 17 cycles (28 S rRNA) at 94 °C for 15 s, 66 °C for 20 s, and 72 °C for 20 s (10 s for 28 S rRNA). The RT-PCR products were quantified after electrophoresis on a 10% polyacrylamide gel and staining (Gelstar, FMC BioProducts) using a Fluor-S™ MultiImager (Bio-Rad).

Real Time Quantitative PCR—Total RNA was isolated from cells 48 h after transfection with siRNA using the High Pure RNA isolation kit (Roche Applied Science). 100 ng of total RNA were reversed transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Real time PCR was performed in a final

volume of 20 μl containing 2 μl of cDNA (corresponding to 10 ng of total RNA for RhoB amplification and corresponding to 0.1 ng of total RNA for glyceraldehyde-3-phosphate dehydrogenase amplification), 300 nM of each primer and 10 μl of the qPCR MasterMix Plus for SYBR® Green (Eurogentec) in the Abi Prism 7000 Sequence Detection system (Applied Biosystems). The results were normalized to the glyceraldehyde-3-phosphate dehydrogenase transcript. PCR was performed with the following primers: RhoB, forward, 5′-GCCACGCGCGCCGCGCTGCA-3′, reverse, 5′-CCGGCAGGGGCAGGCGCGAC-3′; and glyceraldehyde-3-phosphate dehydrogenase: forward, 5′-CCTGGCCAAGGTCATCCATGACA-3′, reverse, 5′-GGGATGACCTTGCCCACAGCCTT-3′.

Creation of the Various Expression Vectors and Cell Transfection—The entire coding sequence of human RhoA was amplified by RT-PCR. The amplification product was mutated by means of a PCR-based approach with mutated primers. Five silent mutations were introduced in the sequence targeted by the 1st siRhoA to make it resistant to this siRNA. The mutated RhoA cDNA (mRhoA) was cloned into pcDNA3 and pcDNA4/TO (Invitrogen). Sequencing confirmed that the five expected mutations were introduced into the cDNA. Rescue experiments with HS578T cells were carried out as previously described (18). Briefly, cells were first transfected with 6 nM siScr or 6 nM siRhoA for 14–16 h following the protocol described above. Immediately after washing, each pool of cells was trypsinized and seeded in two wells of 6-well plates. Three hours later, 1 μg of pcDNA4/TO or pcDNA4/TO/mRhoA were transfected into cells for 20–24 h with 3 μl of Genejuice™ (Novagen). Cells were washed and cultured for a further 24 h before being processed for immunoblotting analysis. Rescue experiments were also carried out with PC-3 cells engineered to express mRhoA in a tetracycline-dependent way. For this purpose, PC-3 cells were first transfected with pcDNA6/TR (Invitrogen) and selected in medium supplemented with 1 μg/ml blasticidin. A clone expressing a high level of tetracycline repressor (PC-3/TR) was isolated. PC-3/TR cells were transfected with either the empty pcDNA4/TO or the pcDNA4/TO/mRhoA and selected in medium supplemented with 1 μg/ml blasticidin + 200 μg/ml Zeocin. 3 clones of cells transfected with pcDNA4/TO (PC-3/TR/control) and 3 clones of cells transfected with pcDNA4/TO/mRhoA (PC-3/TR/mRhoA) were isolated and amplified. For rescue experiments, the PC-3/TR/control and the PC-3/TR/mRhoA clones were seeded at subconfluence in medium without blasticidin and Zeocin. 24 h later, the cells were transfected with 6 nM siScr or 6 nM siRhoA for 14–16 h following the procedure described above. Immediately after washing, each pool of transfected cells was trypsinized and separated in two parts, the first was cultured with normal medium and the second with medium supplemented with 1 μg/ml tetracycline. 48 h later, cells were processed for immunoblotting analysis and a measure of the expression level of both endogenous and mutated RhoA mRNA by RT-PCR. The entire coding sequence of human RhoA was amplified by RT-PCR. A point mutation was introduced in the amplification product by means of a PCR-based approach with mutated primers to generate a cDNA encoding RhoAN19, the dominant negative form of RhoA. The entire sequence was cloned into pcDNA4/TO (Invitrogen). Sequencing confirmed that the expected point mutation was introduced into the cDNA. PC-3/TR cells were transfected with the pcDNA4/TO/RhoAN19 and selected in medium supplemented with 1 μg/ml blasticidin + 200 μg/ml Zeocin. 3 clones (PC-3/TR/RhoAN19) expressing in a tetracycline-dependent way a dominant-negative RhoA were isolated and amplified. The functionality of the dominant-negative RhoA was tested with a GTPase assay as described below. To generate the RhoAR68E mutant, 4 mutations were introduced in the mRhoA cDNA. A HA-tagged RhoB was generated by amplifying the whole human RhoB cDNA with a forward primer including the sequence coding for the HA-FLAG plus a start codon (MYPYDVPDYA) upstream of the first 22 nucleotides of the human RhoB coding sequence and a classical reverse primer hybridizing at the end of the coding sequence. To generate the HA-tagged RhoBR68E, 3 mutations were introduced in the HA-RhoB sequence by a PCR-based approach. These three cDNAs were cloned into pcDNA4/TO. The entire coding sequence of human RhoGDIα was amplified by RT-PCR and cloned into pcDNA3 (pcDNA3_RhoGDIα). The integrity of the four cDNAs was confirmed by sequencing. For plasmid transfection, HS578T cells were seeded in 6-well plates. 3 h after seeding, a total amount of 1 μg of plasmid was transfected into cells for 20–24 h with 3 μl of Genejuice™ (Novagen).

Rho Translocation Assay—A Rho translocation assay was performed as described previously (22, 23). HS578T cells were incubated with lysis buffer containing 50 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 5 mM sodium fluoride, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 4 μg/ml aprotinin, 1 mM dithiothreitol, and 0.1% Triton X-100 for 5 min on ice. The cell lysates were centrifuged at 24,000 × *g* for 15 min at 4 °C. The supernatant corresponding to the cytosol fraction was collected and the pellet resuspended in ice-cold lysis buffer containing 1% Triton X-100 and centrifuged at 24,000 × *g* for 15 min at 4 °C. The supernatant corresponding to the membrane fraction was collected.

GTPase Assays—The assay was carried out as previously described (24, 25). Briefly, cells were chilled on ice and lysed in ice-cold buffer containing 0.5% Triton X-100, 25 mM Hepes, pH 7.3, 150 mM NaCl, 4% glycerol, 10 mM NaF, 20 mM β-glycerophosphate, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 4 μg/ml aprotinin. Lysates were centrifuged for 8 min at 13,000 × *g*. Supernatants were immediately frozen in liquid nitrogen and stored at −80 °C until use. An aliquot of each supernatant collected before freezing was denatured in SDS-PAGE lysis buffer to measure the total RhoGTPase content by Western blotting. For pull-down assays, supernatants were incubated for 30 min with 30 μg of GST-PBD protein containing the Cdc42 and Rac binding region of PAK-1B, or GST-RBD protein containing the Rho binding region of rhotekin affinity linked to glutathione-Sepharose beads. The beads were washed 4 times in lysis buffer and boiled in 60 μl of SDS-PAGE lysis buffer.

Determination of RhoB Protein Stability—48 h after transfection with siRNA or 24 h after transfection with plasmids cells were incubated with 20 μg/ml cycloheximide, at a concentration that effectively blocked synthesis of the RhoB protein (26),

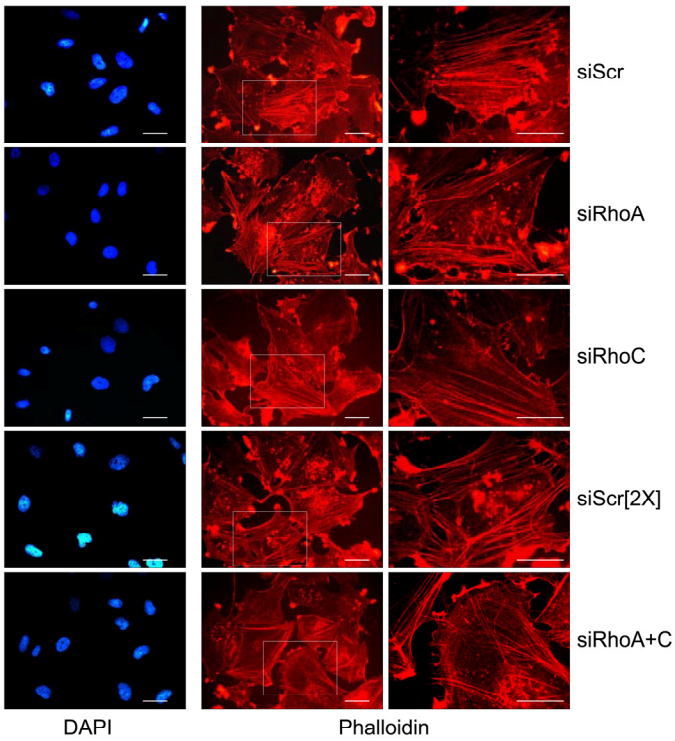

FIGURE 1. **Simultaneousknockdown of RhoA and RhoC did not completely suppress actin stress fibers.** Immediately after transfection with 20 or 40 nM of an irrelevant siRNA (*siScr* or *siScr[2X]*), 20 nM of an siRNA targeting RhoA (*siRhoA*), 20 nM of an siRNA targeting RhoC (*siRhoC*) or 20 nM siRhoA + 20 nM siRhoC (*siRhoA+C*), HS578T cells were seeded on tissue culture dishes. 24 h later, cells were analyzed by fluorescence labeling with phalloidin-TRITC and 4′,6-diamidino-2-phenylindole (*DAPI*). *Bar*, 20 μm.

and the RhoB protein levels were analyzed by Western blot at various time points after cycloheximide addition.

Cytoskeleton Labeling—For fibrillar actin labeling, HS578T cells were fixed with 3% paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. The samples were blocked with 0.2% bovine serum albumin in PBS for 30 min and incubated with 50 ng/ml of phalloidin-TRITC and with 50 ng/ml of 4′,6-diamidino-2-phenylindole for 30 min. Fluorescence was analyzed with a Leica DMRB microscope. A minimum of 100 cells in each condition tested were analyzed to determine the percentage of cells displaying stress fibers.

RESULTS

Silencing RhoA and RhoC Does Not Completely Suppress the Actin Stress Fibers—RhoA is a key determinant in the organization of actin cytoskeleton and cell shape. However, its specific repression, up to 95%, in various cell types with two different sets of siRNA did not alter the cell morphology of either actin stress fibers formation (Refs. 18 and 21 and Fig. 1). According to the data published by Simpson and co-workers (27), this lack of morphological alteration could be due to a compensatory mechanism between RhoA and RhoC. We tested this hypothesis by a double silencing of RhoA and RhoC. Although the morphology of human breast adenocarcinoma cell HS578T was somewhat altered by this double knock-down, stress fibers were still visible, suggesting a residual "Rho-like" activity (Fig. 1). Therefore, we hypothesized that it might be related to the activity of the third member of the Rho subclass, RhoB. This RhoGTPase is indeed able to activate effectors such as ROCK and to induce the formation of actin stress fibers.

RhoA-dependent Regulation of RhoB—RhoB is a short-lived and inducible protein that is barely detectable as observed by Western blot analysis of whole lysate of mock-transfected cells or of cells transfected with a control siRNA (Fig. 2). The knockdown of RhoA in HS578T cells induced a significant up-regulation of the RhoB protein level (Fig. 2). RhoB concentration was also slightly increased upon RhoC silencing, whereas the double silencing of RhoA and RhoC induced its dramatic increase as compared with the single silencing of RhoA (Fig. 2). The negative regulation of RhoB operated by RhoA and RhoC was also observed in human HT1080 fibrosarcoma cells, A2058 melanoma cells, in primary HSF (Fig. 2), and in human prostate PC-3 adenocarcinoma cells (Fig. 4*B*) suggesting that it represents a wide spread mechanism. Similar results were observed by using a second siRNA targeting another sequence of RhoA or RhoC mRNA (Fig. 3*A*). To further validate our observations, we designed control siRNA with two nucleotide changes from the target sequence (CtrRhoA and CtrRhoC) as recommended in Ref. 28. As illustrated in supplementary Fig. S1, the use of these controls confirmed the negative regulations operated by RhoA and RhoC on RhoB but also the specificity of the cross-regulations between RhoA and RhoC. By contrast, the silencing of Rac1 or Cdc42 with our previously published siRNA sequences (18) did not affect RhoB protein level (Fig. 3*A*). The induction of RhoB following RhoA silencing was observed at a concentration of siRNA as low as 0.2 nM (Fig. 3*B*). To definitively ascertain the specificity of the negative regulation operated by RhoA on RhoB expression, the silencing of RhoA was rescued by re-expressing a *RhoA* mRNA resistant to

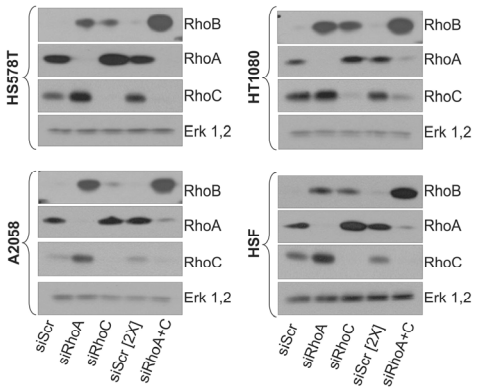

FIGURE 2. **RhoA or/and RhoC silencing significantly increased the RhoB protein level in various cell types.** HS578T cells, A2058 cells, HT1080 cells, or human primary skin fibroblasts (*HSF*) were transfected with 20 or 40 nM of an irrelevant siRNA (*siScr* or siScr[2X]), with 20 nM of an siRNA targeting RhoA (*siRhoA*) or RhoC (*siRhoC*), or with 20 nM of an siRNA targeting RhoA + 20 nM of an siRNA targeting RhoC (*siRhoA+C*). 48 h post-transfection, cells were lysed and analyzed by immunoblotting with specific antibodies to RhoA, RhoB, RhoC, and ERK1,2.

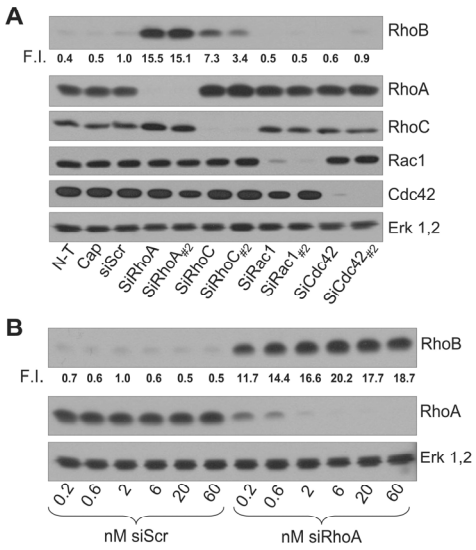

FIGURE 3. **The repression of RhoA or RhoC, but not Rac1 or Cdc42, increased RhoB protein level.** *A*, representative Western blot analysis of cell lysates of HS578T cells untransfected (*NT*) or 48 h after transfection with calcium phosphate alone (*Cap*), 20 nM of an irrelevant siRNA (*siScr*), or 20 nM of two different siRNA targeting RhoA (*siRhoA* or *siRhoA#2*), RhoC (*siRhoC* or *siRhoC#2*), Rac1 (*siRac1* or *siRac1#2*), or Cdc42 (*siCdc42* or *siCdc42#2*). *B*, representative Western blot analysis of HS578T cells 48 h after transfection with the indicated concentrations of siRNA. The factor of induction (*F.I.*) was calculated from densitometric measurements of the RhoB signal normalized to ERK1,2 loading measured compared with the same measurements in the lysates of HS578T cells transfected with siScr in *A* or 2 nM of siScr in *B*.

the first siRNA targeting RhoA by introducing five neutral mutations impairing the silencing by this siRNA (mRhoA) (Fig. 4). HS578T cells were first transfected with scrambled siRNA or the first siRNA targeting RhoA. Immediately after washing and trypsinization, each pool of transfected cells was separated in two halves and transfected either with empty pcDNA3 or pcDNA3/mRhoA as described under "Experimental Procedures." The transient re-expression of RhoA reversed at least partly the overexpression of RhoB (Fig. 4*A*). The residual RhoB (40%) could be due to the presence of some cells that were not transfected by pcDNA3/mRhoA. The strong (more than 10 times the physiological level) and transient (~24 h) re-expression of RhoA is obviously not the most appropriate manner to rescue silencing. To reverse more adequately RhoA silencing, we transfected PC-3 cells stably expressing a repressor sensitive to tetracycline (PC-3/TR) with an empty inducible vector (pcDNA4/TO) or with the same vector encoding mRhoA (pcDNA4/TO/mRhoA) to generate, respectively, control clones (PC-3/TR/control) or clones expressing mRhoA in a tetracycline-dependent way (PC-3/TR/mRhoA). Three clones of PC-3/TR/control cells and 3 clones of PC-3/TR/mRhoA cells were isolated. These clones were transfected with 6 nM scrambled siRNA or 6 nM siRhoA. Immediately after washing and trypsinization, each pool of transfected cells was separated in two halves and cultured for 2 days either in the absence or presence of 1 μg/ml tetracycline. Cells were then processed for Western blot or RT-PCR analysis (Fig. 4*B*). RT-PCR analysis revealed that endogenous RhoA was similarly repressed in the absence or presence of tetracycline, whereas the mutated RhoA was significantly induced by tetracycline. The re-expression of the RhoA protein in these conditions nearly completely reversed the overexpression of RhoB, whereas treatment of PC-3/TR/control cells with tetracycline did not affect the RhoB protein level. These results definitively confirm the negative regulation operated by RhoA on RhoB. It should be noted that the re-expression of RhoA also reversed the up-regulation of RhoC upon RhoA silencing (supplementary Fig. S2).

The Up-regulated RhoB Is Biologically Active—As measured by pull-down assay, the increased RhoB level observed in RhoA and/or RhoC silenced cells is paralleled by an enhanced level of the RhoB active form (Fig. 5*A*). RhoB-GTP was estimated to represent between 2 and 3% of the total up-regulated RhoB. However, an efficient activation of the downstream effectors also requires translocation of the RhoGTPase to the membrane. To evaluate the subcellular localization of RhoB, a differential extraction procedure was used. It showed that up-regulated RhoB is mainly associated with the membrane fraction (Fig. 5*B*). These observations suggested that the induced RhoB was functional and could be responsible for the residual Rho-like activity observed in Fig. 1. To address this issue, a simultaneous repression of RhoA, RhoC, and RhoB was performed in HS578T cells. Western blot analysis and pull-down assays collected 48 h after transfection supported the efficient repression of total and active RhoB (Fig. 5*A*). The simultaneous knockdown of RhoA, RhoB, and RhoC effectively suppressed the actin stress fibers network as compared with its maintenance in

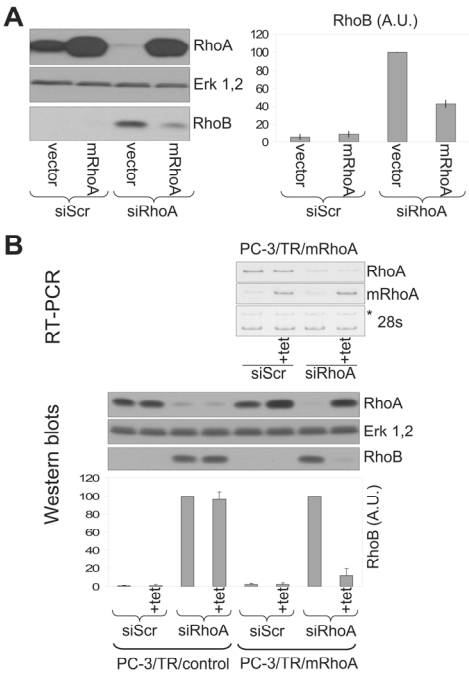

FIGURE 4. **The rescue of RhoA silencing reverses RhoB overexpression.** *A,* representative Western blot analysis with specific antibodies to RhoA, RhoB, or ERK1,2 of whole cell lysates of HS578T transfected with 6 nM of an irrelevant siRNA (*siScr*) or 6 nM of the first siRNA targeting RhoA (*siRhoA*) and 1 μg of empty pcDNA3 (*vector*) or pcDNA3/mRhoA (*mRhoA*). The *right panel* illustrates the densitometric analysis of RhoB signal intensity. Results are the mean ± S.D. of three independent experiments. *B*, PC-3/TR/control and PC-3/TR/mRhoA cells were transfected with 6 nM of an irrelevant siRNA (*siScr*) or 6 nM of the first siRNA targeting RhoA (*siRhoA*). Cells were supplemented (+*tet*) or not with 1 μg/ml of tetracycline for 48 h and processed for RT-PCR analysis (*top panel*) or Western blot (*middle panel*). Representative analyses of six independent experiments performed on 3 clones of PC-3/TR/control and 3 clones of PC-3/TR/mRhoA are illustrated. The *bottom panel* illustrates the mean ± S.D. of densitometric analysis of RhoB protein measurements. *AU*, arbitrary units.

the double knockdown RhoA + RhoC (Fig. 5*C*). The percentage of HS578T cells displaying stress fiber was 89 ± 3% in cells transfected with siScr, 69 ± 2% in cells transfected with siRhoA + siRhoC, and 25 ± 2% in cells transfected with siRhoA + siRhoC + siRhoB.

RhoB Up-regulation Is Related to a Prolonged Half-life of the Protein—As assessed by real time quantitative PCR measurements following RhoA or RhoC silencing, the induction of RhoB was not related to an increase of its mRNA level (Fig. 6*A*). It was, however, clearly up-regulated by the double silencing RhoA + RhoC. The stability of the RhoB protein was determined in HS578T cells by blocking protein synthesis with cycloheximide. As compared with cells transfected with the scrambled siRNA, the half-life of RhoB was slightly increased in RhoC-silenced cells. By contrast, RhoA silencing as well as the double silencing RhoA + RhoC induced a dramatic increase of RhoB stability from 3.0 to 16.5 h (Fig. 6*B* and Table 1). In the double knock-down RhoA + RhoC, the RhoB protein level is therefore up-regulated both by transcriptional and post-translational mechanisms. Similar results were also obtained in HSF (not shown).

The Induction of RhoB Is Neither Dependent on Isoprenoid Biosynthesis, nor ERK1,2, p38MAPK, or PI3K Pathways, nor ROCK or RhoA Activity—Inhibitors of the mevalonate pathway such as simvastatin have been reported to increase the stability of RhoB by reducing the availability of isoprenoid intermediates required for translocation to the membrane (26). It was also observed in our models (Fig. 5*B*). Addition of geranylgeranylpyrophosphate and/or farnesyl-pyrophosphate rescued the simvastatin-induced up-regulation of RhoB (26). A similar procedure used in RhoA- and/or RhoC-silenced cells did not alter the up-regulation of RhoB (supplementary Fig. S3). Moreover, by using a differential extraction procedure, we observed that up-regulated RhoB by silencing RhoA was associated with the membrane fraction, whereas the RhoB induced upon simvastatin treatment was cytoplasmic (Fig. 5*B*). These results rule out the implication of isoprenoid biosynthesis in the up-regulation of RhoB reported here. In parallel, we tested the involvement of the ERK1,2, p38MAPK, and PI3K pathways by means of pharmacological inhibitors. Used at concentrations allowing an efficient inhibition of their specific target, none of these inhibitors affected the RhoA-dependent regulation of RhoB (supplemental Fig. S3) suggesting that neither ERK1,2, p38MAPK, nor PI3K pathways were involved in the up-regulation of RhoB reported here. Alternatively, the knockdown of RhoA and/or RhoC could affect the activation level of a common downstream effector such as ROCK. To test this hypothesis, ROCK was specifically inhibited in HS578T cells with daily renewed Y-27632 during 48 h of culture. This treatment barely modulated the RhoB protein level in I IS578T cells (Fig. 7*A*), suggesting that the RhoA-mediated RhoB regulation did not depend on the classical activation of downstream signaling cascades upon GTP loading. A siRNA approach as we used depletes both GTP- and GDP-bound RhoA. To discriminate between the effects of both forms, PC-3 cell lines expressing, in an inducible way, the dominant-negative form of RhoA were created by transfecting PC-3 cells stably expressing a repressor sensitive to tetracycline (PC-3/TR) with a vector encoding RhoAN19 (pcDNA4/TO/RhoAN19) that prevents GDP-GTP exchange. Three clones of PC-3/TR/RhoAN19 cells were isolated on the basis of their ability to express upon tetracycline addition a functional RhoAN19 by using a pull-down assay (Fig. 7). RhoAN19 is visible as a slower migrating band in Fig. 7. As observed in Fig. 7*B*, the repression of RhoA-GTP alone was not sufficient to increase RhoB expression. By contrast, the silencing of RhoA in the three clones of PC-3/TR/RhoAN19 induced RhoB expression as efficiently with (+tet) or without (−tet) prior depletion of RhoA-GTP (Fig. 7*C*). These results suggest that the regulation of RhoB expression is mediated by RhoA-GDP and not by RhoA-GTP.

RhoGDIα Is Involved in the Induction of RhoB—Several studies suggested that in addition to regulating RhoGTPase activation, RhoGDIs protect some RhoGTPases from degradation

RhoA-GDP Regulates RhoB

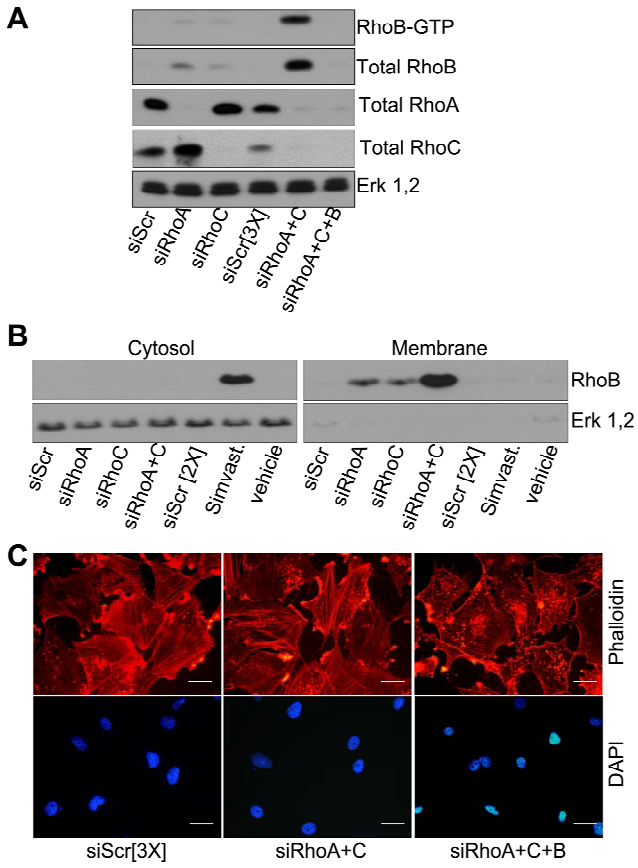

FIGURE 5. **RhoB induced by silencing RhoA and/or RhoC is active and associated with the membrane fraction.** *A*, HS578T cells were processed for pull-down and Western blot analysis with specific antibodies to RhoA, RhoB, and RhoC 48 h after transfection with 20 nM of an irrelevant siRNA (*siScr*), the first siRNA targeting RhoA (*siRhoA*), or the first siRNA targeting RhoC (*siRhoC*), with 60 nM siScr (*siScr*[*3X*]), 20 nM siRhoA + 20 nM siRhoC + 20 nM siScr (*siRhoA*+*C*), or 20 nM siRhoA + 20 nM siRhoC + 20 nM siRhoB (*siRhoA*+*C*+*B*). *B*, the cytosolic and membrane fractions of HS578T cells transfected with 20 or 40 nM of an irrelevant siRNA (*siScr* or *siScr*[*2X*]), with 20 nM of the first siRNA targeting RhoA (*siRhoA*) or RhoC (*siRhoC*), or with 20 nM of the first siRNA targeting RhoA + 20 nM of the first siRNA targeting RhoC (*siRhoA*+*C*), or treated with 10 μM simvastatin (*simvast.*) or ethanol alone (*vehicle*) were obtained as described under "Experimental Procedures." Both fractions (10 μg) were analyzed by Western blot with specific antibodies against RhoB and ERK1,2. *C*, immediately, after transfection with 60 nM siScr (*siScr*[*3X*]), 20 nM siRhoA + 20 nM siRhoC + 20 nM siScr (*siRhoA*+*C*), or 20 nM siRhoA + 20 nM siRhoC + 20 nM siRhoB (*siRhoA*+*C*+*B*), HS578T cells were seeded on tissue culture dishes for 24 h and analyzed by fluorescence labeling by phalloidin-TRITC and with 4′,6-diamidino-2-phenylindole (*DAPI*). *Bar*, 20 μm.

(7). To address the role of RhoGDIα in RhoB protein stability we transfected HS578T cells with an expression vector encoding the whole coding sequence of human RhoGDIα. Its forced expression strongly enhanced the RhoB protein level as compared with cells transfected with an empty vector where RhoB was barely detectable (Fig. 8*A*). Upon RhoGDIα overexpression, RhoB was mainly membrane-bound (supplementary Fig. S4*B*). The GTP-bound RhoB form was also increased and represented between 2 and 3% of total RhoB (supplementary Fig. S4*A*). This up-regulation of RhoB was associated with a strong increase of its half-life (Table 1) suggesting that RhoGDIα may protect RhoB from degradation (Fig. 8*B*). This potential role was tested by performing simultaneous knockdown of RhoA and RhoGDIα in HS578T cells. The level of RhoB protein 48 h after transfection with the two different siRNA targeting RhoA alone was reduced by more than 70% if RhoGDIα was simultaneously knocked down (Fig. 8*C*). To investigate more directly the involvement of RhoGDIα in the regulation of RhoB, we generated by a PCR-based approach a cDNA encoding a RhoA mutant (mRhoAR68E) unable to bind RhoGDIα but still able to undergo GDP-GTP exchange and to bind downstream effectors as assessed by a pull-down assay (supplementary Fig. S5). The construct further contains five silent mutations making the encoded mRNA resistant to the first siRhoA as shown in rescue experiments in Fig. 8*D*. Even though this mRhoAR68E mutant was strongly expressed it was significantly less efficient as compared with wild-type RhoA in rescuing RhoB up-regulation upon RhoA silencing (Fig. 8*D*). To further investigate the molecular mechanism driving RhoB stabilization, we tested its interaction with RhoGDIα. Co-immunoprecipitation experiments in siRhoA-transfected cells did not reveal any association between endogenous RhoB and RhoGDIα. A direct interaction between RhoB and RhoGDIα could be observed by co-immunoprecipitation in lysates of HS578T cells co-transfected with expression vectors encoding a HA-tagged RhoB and RhoGDIα (supplemental Fig. S6). The generation of the R68E mutation in RhoB completely abolished its interaction with RhoGDIα (supplemental Fig. S6). Co-transfection of RhoGDIα increased significantly the half-life of HA-tagged RhoB but not that of HA-tagged RhoBR68E (Table 1). Altogether, these results suggest that RhoGDIα is involved in the RhoA-mediated regulation of RhoB. Although an interaction between RhoB and RhoGDIα

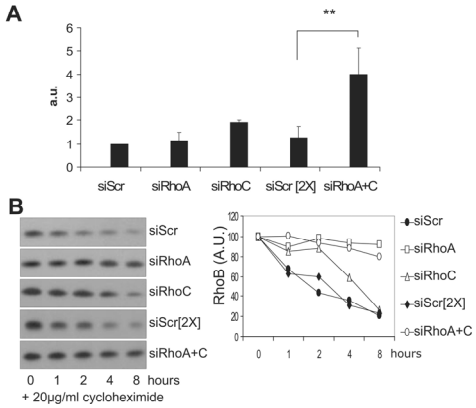

FIGURE 6. **The up-regulation of RhoB by silencing RhoA or RhoC was related to an increased half-life of the protein.** *A*, real time quantitative PCR analysis of the RhoB mRNA level was performed with total RNA extracted from HSF cells 48 h after transfection with an irrelevant siRNA at 20 or 40 nM (*siScr* or *siScr[2X]*), 20 nM of the first siRNA targeting RhoA (*siRhoA*), 20 nM of the first siRNA targeting RhoC (*siRhoC*), or 20 nM of the first siRNA targeting RhoA + 20 nM the first siRNA targeting RhoC (*siRhoA+C*). Results are the mean ± S.D. of three independent experiments. **, $p < 0.01$ analysis of variance followed by Tukey-Kramer analysis. *B*, HS578T cells were transfected with 20 or 40 nM of an irrelevant siRNA (*siScr* or *siScr[2X]*), 20 nM of the first siRNA targeting RhoA (*siRhoA*) or RhoC (*siRhoC*), or 20 nM siRhoA + 20 nM siRhoC (*siRhoA+C*). 48 h after transfection, cells were supplemented with 20 μg/ml cycloheximide for the indicated time before being processed for Western blot analysis. Representative blots of three independent experiments are shown. The *right panel* illustrates the densitometric analysis of the illustrated blots. It has to be noted that blots were loaded with 30 (*siScr* and *siScr[2X]*), 10 (*siRhoC*), or 5 μg of proteins (*siRhoA* and *siRhoA+C*) to obtain initial similar signal intensities.

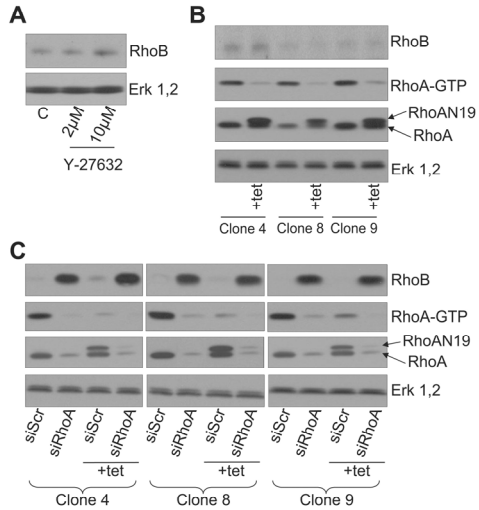

FIGURE 7. **The regulation of RhoB is mediated by RhoA-GDP.** *A*, HS578T cells were cultured for 2 days with vehicle alone (*c*) or the indicated concentrations of ROCK inhibitor Y-27632, renewed daily, and processed for Western blot analysis with specific antibodies to RhoB and ERK1,2. *B*, 3 clones of PC-3/TR/RhoAN19 cells were supplemented (+*tet*) or not with 1 μg/ml of tetracycline for 48 h and processed for RhoA pull-down assay and Western blot analysis with specific antibodies to RhoA, RhoB, and ERK1,2. Representative analyses are illustrated. *C*, after 48 h of culture with (+*tet*) or without 1 μg/ml tetracycline, the 3 clones of PC-3/TR/RhoAN19 were transfected with 6 nM of an irrelevant siRNA (*siScr*) or 6 nM of the first siRNA targeting RhoA (*siRhoA*). After washing, cells were cultured for 48 more hours and then processed for RhoA pull-down assay and Western blot analysis with specific antibodies to RhoA, RhoB, and ERK1,2. Where indicated, the tetracycline was maintained in the culture medium throughout the experiment. The *upper band* observed in the "Total RhoA" blots corresponds to the dominant-negative RhoA (*RhoAN19*).

TABLE 1

Wild type and mutated RhoB stability

RhoB, HA-RhoB, and HA-RhoBR68E protein half-life were measured in HS578T cells after transfection with the indicated siRNA or plasmids.

Transfection conditions	RhoB half-life
siRNA	
Scr	3.0 ± 0.9 h
siRhoA	16.3 ± 4.4 h
siRhoC	5.1 ± 1.6 h
scr (40 nM)	3.7 ± 0.8 h
siRhoA + siRhoC	16.5 ± 1.0 h
Plasmids	
Empty pcDNA3	2.1 ± 0.6 h
pcDNA3_RhoGDIα	13.5 ± 3.5 h
HA-RhoB half-life	
pcDNA4_HA-RhoB + empty pcDNA3	2.5 ± 0.1 h
pcDNA4_HA-RhoB + pcDNA3_RhoGDIα	4.6 ± 0.3 h
HA-RhoBR68E half-life	
pcDNA4_HA-RhoBR68E + empty pcDNA3	4.9 ± 1.5 h
pcDNA4_HA-RhoBR68E + pcDNA3_RhoGDIα	3.6 ± 1.3 h

can be observed in overexpression experiments, a direct role for RhoGDIα in the stabilization of endogenous RhoB remains elusive.

DISCUSSION

Deciphering the functions and regulations of closely related members of the RhoGTPase family like RhoA, RhoB, and RhoC requires a highly specific approach. In this study, we took advantage of the high precision afforded by the siRNA technology to underscore the role played by mainly RhoA, and to a lesser extent by RhoC, in the regulation of RhoB. The control operated by RhoA on RhoB appears to be a widespread mechanism as it was observed in many cell types of various lineages. The induction of RhoB protein with two different siRNA targeting RhoA and at concentrations as low as 0.2 nM are significant arguments supporting the specificity of our observations. Rescue experiments with a mutated RhoA mRNA (mRhoA) resistant to the first siRNA targeting RhoA definitely validate our results. To appropriately rescue the effect of RhoA silencing, we generated clones of PC-3 cells expressing the mRhoA in a tetracycline dependent way. The concentration of tetracycline used here (1 μg/ml) did not affect the RhoA-dependent regulation of RhoB as observed in the control clones. The inducible re-expression of mRhoA was close to the physiological range in the 3 PC-3/TR/mRhoA clones tested and was maintained as long as tetracycline was present. By using this procedure, the rescue nearly completely suppressed the up-regulation of RhoB. This technology also opens the way to *in vivo* rescue experiments by feeding animals, in which these cells could be injected to

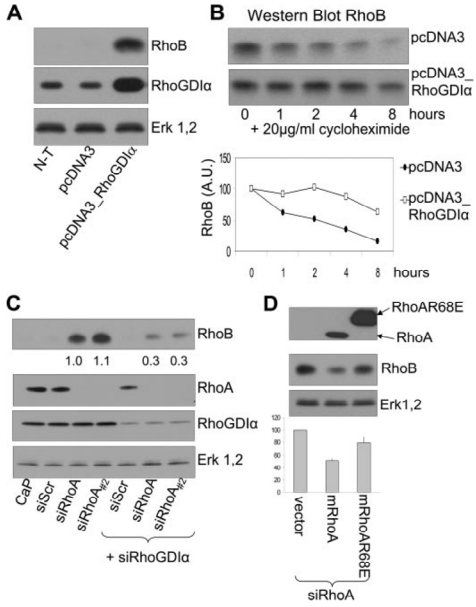

FIGURE 8. **RhoB up-regulation depends on RhoGDIα availability and a RhoA mutant unable to bind RhoGDIα is less efficient than WT-RhoA in rescuing RhoB up-regulation.** *A*, HS578T cells were left untransfected (*N-T*), transfected with an empty vector (*pcDNA3*), or transfected with an expression vector encoding the whole coding cDNA sequence of RhoGDIα (*pcDNA3_RhoGDIα*). 24 h after transfection, cells were processed for Western blot analysis with specific antibodies to RhoB, RhoGDIα, and ERK1,2. *B*, HS578T cells were transfected with an empty vector (*pcDNA3*) or with an expression vector encoding the whole coding DNA sequence of RhoGDIα (*pcDNA3_RhoGDIα*). 24 h after transfection, cells were cultured with 20 μg/ml cycloheximide for the indicated time before being processed for Western blot analysis. Representative blots of three independent experiments are shown. The *bottom panel* illustrates the densitometric analysis of the illustrated blots. Blots were loaded with 30 (*pcDNA3*) or 5 μg (pcDNA3_RhoGDIα) of proteins to obtain similar initial signal intensities. *C*, HS578T cells were transfected with calcium phosphate alone (*CaP*), 20 nM of an irrelevant siRNA (*siScr*), 20 nM of the first or of the second siRNA targeting RhoA (*siRhoA* or *siRhoA#2*) alone, or in combination with 20 nM siRhoGDIα (+*siRhoGDIα*). 48 h after transfection, cells were processed for Western blot analysis with specific antibodies to RhoB, RhoA, RhoGDIα, and ERK1,2. *D*, representative Western blot analysis with specific antibodies to RhoA, RhoB, or ERK1,2 of whole cell lysates of HS578T transfected with 10 nM of the first siRNA targeting RhoA (*siRhoA*) and 1 μg of empty pcDNA4/TO (*vector*), pcDNA4/TO/mRhoA (*mRhoA*), or pcDNA4/TO/mRhoAR68E (*mRhoAR68E*). The *lower panel* illustrates the densitometric analysis of RhoB signal intensity. Results are the mean ± S.D. of three independent experiments.

evaluate, for instance, their tumorigenic potential, with stable tetracycline analogs, like doxycycline.

No modulation of the RhoB mRNA level in RhoA-silenced cells was observed by real time quantitative PCR analysis. Moreover, two independent comparisons of the gene expression profiles by microarrays of PC-3 cells transfected either with an irrelevant siRNA or the first siRNA targeting RhoA did not reveal any modulation of RhoB mRNA expression.[5] We demonstrated that the increase of RhoB occurs at a post-transcriptional level through stabilization of the protein as assessed by half-life measurements. Fritz and Kaina (29) reported an up-regulation of the *RhoB* gene by overexpressing the dominant-negative mutant RhoAN19. The results reported here demonstrate that silencing of a RhoGTPase does not simply mimic the overexpression of a dominant-negative mutant that targets guanine-nucleotide exchange factors. Whereas some Rho guanine-nucleotide exchange factors are highly specific, others activate multiple Rho GTPases (30). Our data suggest that the double silencing of RhoA + RhoC is an experimental condition closer to the overexpression of RhoAN19 than the single silencing of RhoA. This is likely because the RhoA dominant-negative mutant depletes the pool of guanine-nucleotide exchange factor(s) that activate both RhoA and RhoC. Our results clearly demonstrate that, under physiological conditions, RhoA is the main determinant to set the low steady-state level of RhoB through (a) post-transcriptional(s) mechanism(s) that strongly decrease(s) the half-life of the protein. The RhoA-mediated regulation of RhoB has a physiological significance and can be operational in cells exposed to bacterial toxins such as *Clostridium difficile* toxin B (TcdB). Genth *et al.* (31) reported that, in cells treated with 1 ng/ml of TcdB, the RhoA level was decreased with a parallel increase in the RhoB level. More recently, Huelsenbeck *et al.* (32) demonstrated that the apoptotic effect of TcdB was mediated by RhoB.

Our results strongly suggest that the RhoA-dependent regulation of RhoB does not depend on the activity of RhoA but is mediated by its GDP-bound form explaining why such a regulation is only visible upon depletion of the inactive pool of RhoA. Using a siRNA approach, Simpson *et al.* (27) recently reported a cross-regulation between RhoA and RhoC that was also observed in our models. Thus, our results suggest that such regulations, uncovered by a siRNA-based approach and undetectable by conventional tools, are common in the RhoGTPase family.

By contrast to RhoA and RhoC reported to be up-regulated in various cancers, RhoB displays properties that might participate in tumor suppression (33). As assessed by the pull-down assay and indicated by the presence of actin stress fibers the up-regulated RhoB is indeed actually biologically active. It could contribute to the anti-tumoral effect of siRNA targeting RhoA or RhoC recently reported *in vitro* and *in vivo* (34). Furthermore, we observed that the induction of the cell cycle inhibitor $p21^{CIP1}$ following RhoC silencing in PC-3 cells is RhoB-dependent.[5]

It is well documented that alterations of post-translational prenylation of RhoB by inhibitors of the mevalonate pathways or farnesyl transferase can increase the expression of RhoB by acting at a transcriptional level (35) but also through modulation of the protein stability (26). However, the RhoB protein induced by treatment with one of these inhibitors, simvastatin as we showed here, is cytosolic, whereas the RhoB induced by repressing RhoA is associated with the membrane fraction similarly to the RhoB expressed in basal conditions. This suggests that silencing of RhoA did not affect post-translational lipid modifications required for RhoB subcellular localization. Moreover, the increased half-life of RhoB following RhoA

[5] T. T. G. Ho, C. M. Lapière, B. V. Nusgens, and C. F. Deroanne, unpublished data.

silencing was not reversed by supplementation of prenylation precursors such as geranylgeranyl-pyrophosphate or farnesyl-pyrophosphate demonstrating that the mevalonate pathway is not involved in the regulation of RhoB in our experimental model. Nevertheless, isoprenylation might constitute a signal for RhoB degradation (26). In physiological conditions, the isoprenyl moiety is masked upon binding to RhoGDIs (36) thus explaining, at least in part, how RhoGDI can protect the RhoGTPases from ubiquitin-mediated degradation as reported for RhoA (7).

RhoGDIγ has been reported to be the preferred RhoGDI partner of RhoB (37, 38). However, similarly to RhoGDIβ, its pattern of expression is tissue specific and is unlikely to account for the RhoA-dependent regulation of RhoB, a process observed in many cell types derived from various tissues (37). We considered that the ubiquitously expressed RhoGDIα was a better candidate. Moreover, it was described as interacting with RhoB in some studies (39). Our data strongly suggest that RhoGDIα can stabilize RhoB and is operational in the up-regulation of RhoB following RhoA silencing. Moreover, we observed that the ability of RhoA to bind RhoGDIα is involved, at least partly, in the regulation of RhoB reported here. The affinity of RhoGDIα is likely higher for RhoA and RhoC than for RhoB because the latter lacks Ser-188, a residue affecting positively the binding to RhoGDIα upon phosphorylation by PKA (7, 40). In overexpression experiments, an interaction between RhoB and RhoGDIα was actually observed. Moreover, transfected RhoGDIα can stabilize wild-type RhoB and not RhoBR68E that has lost the ability to bind RhoGDIα. However, a direct stabilization of endogenous RhoB by RhoGDIα remains elusive. Alternatively, it could involve the interaction of RhoGDIα with some proteins other than RhoB. Quantitation of the RhoGDIα level in various cell types shows that its molar amount is roughly equal to the molar amount of the three GTPases: RhoA, Rac1, and Cdc42 (38). In physiological conditions RhoGDIα is thus likely rate-limiting and its overexpression or the silencing of RhoA are two means for making it available to stabilize RhoB. The freed RhoGDIα by RhoA silencing could also contribute to the up-regulation of the RhoC protein level that we and others observed upon RhoA depletion (Fig. 2 and Ref. 27). In agreement with our hypothesis, it should be noticed that TcdB prevents RhoA from interacting with RhoGDI (32) and, as mentioned above, increases in parallel the RhoB protein level. Recently, Wong and co-workers (10) reported that the alteration of RhoGDI-dependent cross-talk between RhoA and Rac1 suppresses integrin-mediated bacterial uptake. Altogether these data suggest that such interplays should be a widespread mechanism to control the stability and the activity of the RhoGTPases.

Our study demonstrates that silencing a RhoGTPase does not simply recapitulate the effects of a dominant-negative mutant but reveal novel mechanisms of regulation. The identification of these mechanisms is related to the highest specificity of the siRNA approach but also to the strategy of depletion of both GTP- and GDP-bound forms thus unraveling an unexpected role for RhoA-GDP. Mechanisms implicating the GDP-bound form of RhoGTPase are not restricted to RhoA. Arozarena and co-workers (2) previously reported the involvement of Cdc42-GDP in Ras signaling. Di-Poi and co-workers (9) observed that Rac1-GDP in complex with RhoGDI can efficiently activate the NADPH oxidase. Our results also suggest that RhoGDIα is a necessary component for stabilizing RhoB that could act directly, indirectly, or as part of a multiproteic complex. The potential interplays between members of the RhoGTPase family should be taken into account for analysis of loss-of-function experiments as well as for the efficient design of therapeutic strategies based on a siRNA approach.

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SUPPLEMENTARY FIGURE LEGENDS

Fig.S1. The use of specific controls for RhoA and RhoC silencing confirms the cross-regulations between the three members of the Rho sub-class. HS578T cells were transfected with 20 nM of the specific control for siRhoA (CtrRhoA), 20nM of the first siRNA targeting RhoA (siRhoA), 20nM of the specific control for siRhoC (CtrRhoC), 20nM of the first siRNA targeting RhoC (siRhoC), 20nM of CtrRhoA+20nM of CtrRhoC (CtrRhoA+C) or 20nM of siRhoA+ 20nM of siRhoC (SiRhoA+C). 24 hours after transfection, the cells were processed for Western blot analysis with specific antibodies to RhoA, RhoB, RhoC or Erk1/2.

Fig.S2. The rescue of RhoA silencing reverses RhoC over-expression. PC-3/TR/control and PC-3/TR/mRhoA cells were transfected with 6 nM of an irrelevant siRNA (siScr) or 6 nM of the first siRNA targeting RhoA (siRhoA). Cells were supplemented (+tet) or not with 1 µg/ml of tetracycline for 48 hours and processed for Western blot analysis with specific antibodies to RhoA, RhoC or Erk1,2.

Fig.S3. The stabilization of RhoB is not dependent on isoprenoid biosynthesis, Erk1,2, p38MAPK or PI3K pathways. HS578T cells were treated with 10µM U0126, 10µM SB203580, 25µM LY294 or 0.1% DMSO (vehicle) alone. After 24 hours, cells were processed for Western blot analysis with specific antibodies to Erk1,2, Phospho-Erk1,2, p38, Phospho-P38, AKT and Phospho-AKT to assess the efficiency of the pharmacological inhibitors (*A*). HS578T cells transfected with 20 nM of an irrelevant siRNA (siScr) or 20 nM of the first siRNA targeting RhoA (siRhoA). Immediately after transfection, cells were cultured with (*B)* 10µM U0126, 10µM SB203580, 25 µM LY294 or 0.1% DMSO (vehicule) or (*C*) with 5µM farnesylpyrophosphate (Fpp), 5µM geranylgeranylpyrophosphate (Gpp), 5µM geranylgeranylpyrophosphate+ 5µM farnesylpyrophosphate (Gpp+Fpp), 0.1% methanol (vehicule) or 0.2% methanol (vehicule[2X]). 24 hours after transfection, cells were processed for Western blot analysis with specific antibodies to RhoB, RhoA and Erk1,2.

Fig.S4. RhoGDIα over-expression increased total and GTP-bound RhoB which is membrane-associated. HS578T cells were transfected with 1µg of pcDNA3 or 1µg of pcDNA3_RhoGDIα. 24 hours after transfection, cells were processed for Western blot analysis with specific antibodies to RhoB, RhoGDIα and Erk1,2 (*A*) or for collecting the cytosolic and membrane fractions as described in Experimental Procedures (*B*). Both fractions (10 µg) were analyzed by immunoblotting with specific antibodies against RhoB and Erk1,2.

Fig.S5. The mutant protein RhoAR68E is unable to associate with RhoGDIα but still able to undergo GDP-GTP exchange. HS578T cells were transfected with 10 nM of the first siRNA targeting RhoA (siRhoA) and 1 µg of empty pcDNA4/TO (vector), pcDNA4/TO/mRhoA (mRhoA) or pcDNA4/TO/mRhoAR68E (mRhoAR68E). 24 hours after transfection, the cells were processed for RhoA pull down assay (RBD pull down), for RhoGDIα immunoprecipitation (IP RhoGDIα) and for Western blot analysis with specific antibodies to RhoA, RhoGDIα or Erk1,2.

Fig.S6. RhoB wild type but not the mutant protein RhoBR68E is able to associate with RhoGDIα. HS578T cells were seeded in 10-cm dishes and transfected with 3 µg of pcDNA4/TO/HA-RhoB + 3 µg of pcDNA3/RhoGDIα or with 3 µg of pcDNA4/TO/HA-RhoBR68E + 3 µg of pcDNA3/RhoGDIα. 24 hours after transfection, the cells were processed for immunoprecipitation with 2µg of anti-RhoGDIα antibodies (IP RhoGDIα) or with 2µg normal rabbit IgG (Control IgG) and for Western blot analysis with specific antibodies to HA-tag or RhoGDIα.

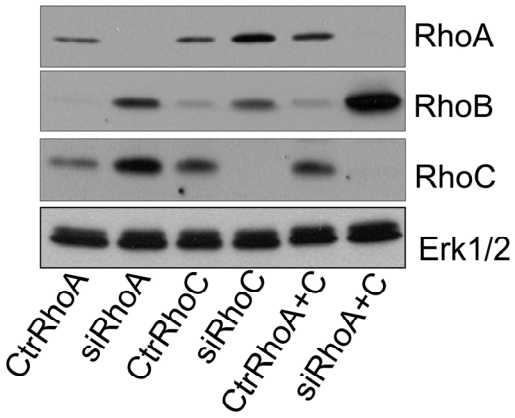

RhoA
RhoB
RhoC
Erk1/2
CtrRhoA
siRhoA
CtrRhoC
siRhoC
CtrRhoA+C
siRhoA+C

Fig.S1

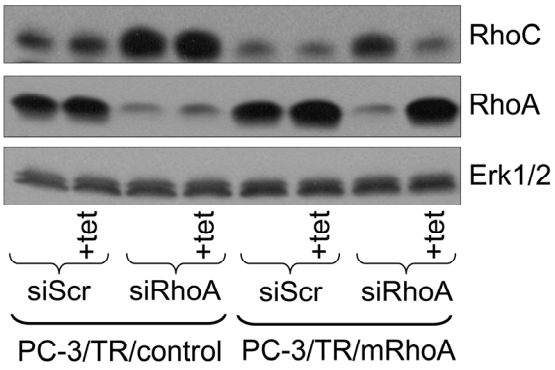
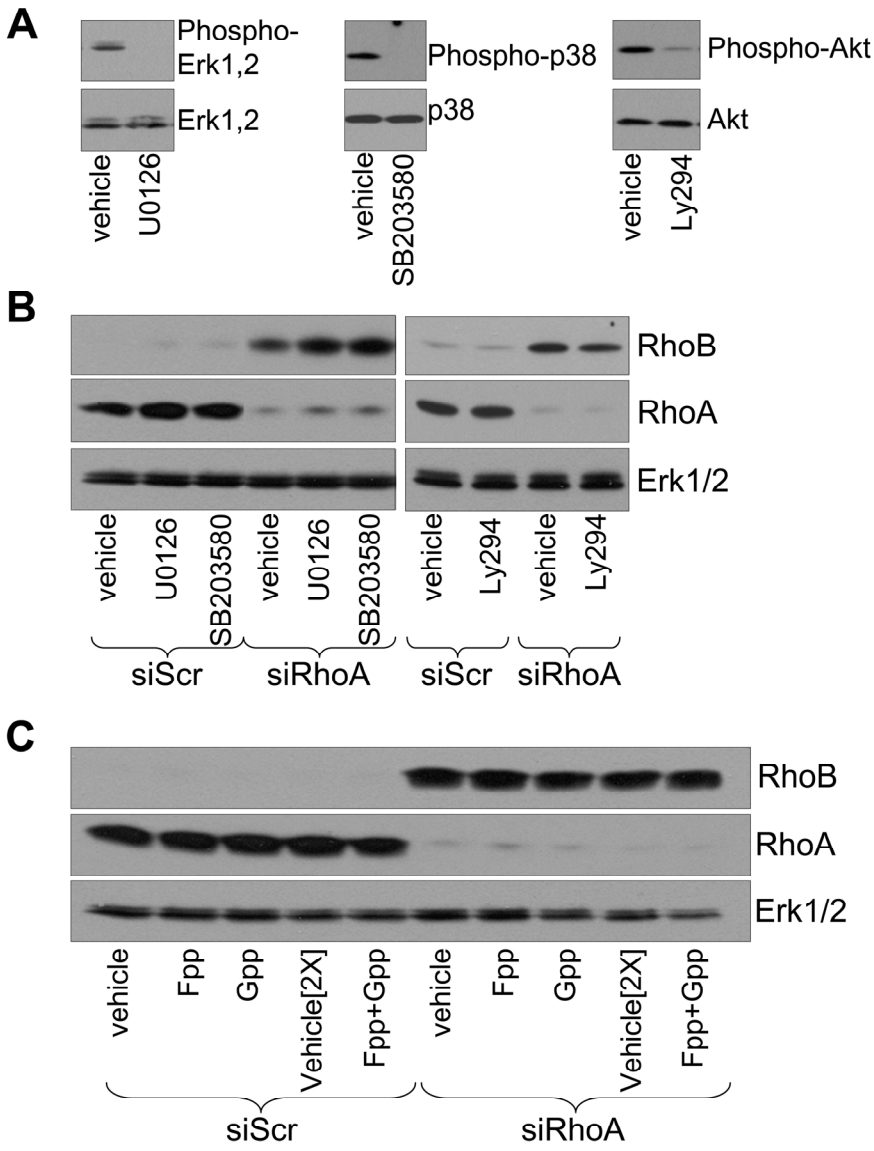

RhoC
RhoA
Erk1/2
+tet
+tet
+tet
+tet
siScr
siRhoA
siScr
siRhoA
PC-3/TR/control
PC-3/TR/mRhoA

Fig.S2


A
Phospho-Erk1,2
Erk1,2
vehicle
U0126
Phospho-p38
p38
vehicle
SB203580
Phospho-Akt
Akt
vehicle
Ly294
B
RhoB
RhoA
Erk1/2
vehicle
U0126
SB203580
vehicle
U0126
SB203580
vehicle
Ly294
vehicle
Ly294
siScr
siRhoA
siScr
siRhoA
C
RhoB
RhoA
Erk1/2
vehicle
Fpp
Gpp
Vehicle[2X]
Fpp+Gpp
vehicle
Fpp
Gpp
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Fpp+Gpp
siScr
siRhoA

Fig.S3

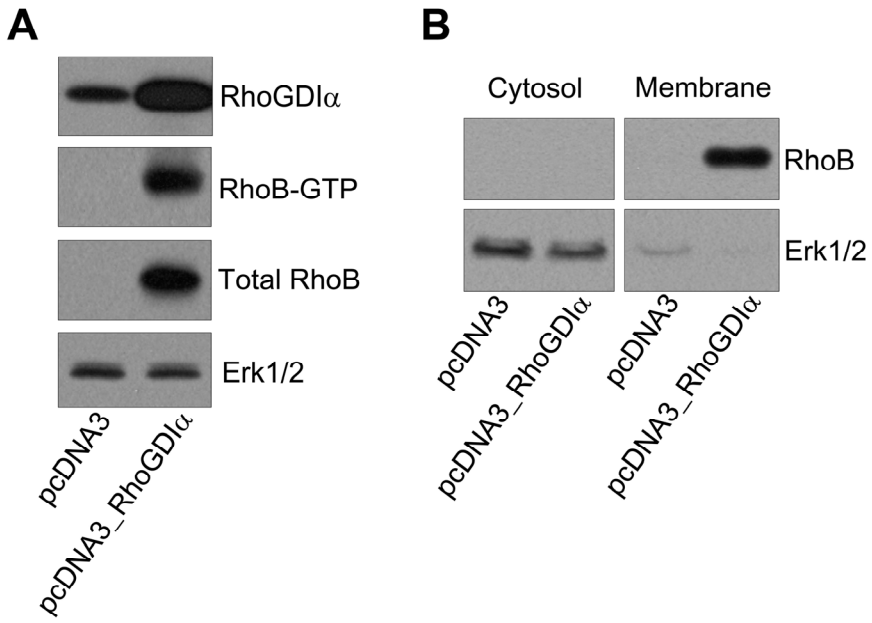
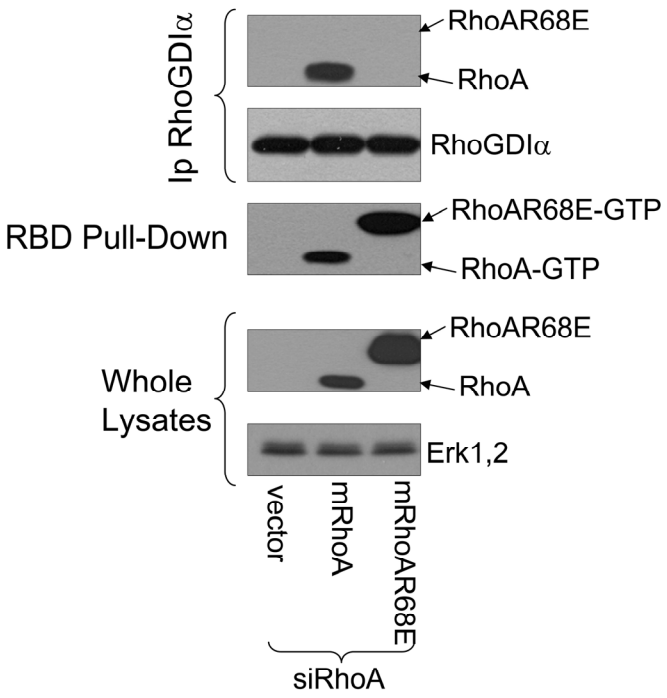

A
RhoGDIα
RhoB-GTP
Total RhoB
Erk1/2
pcDNA3
pcDNA3_RhoGDIα
B
Cytosol
Membrane
RhoB
Erk1/2
pcDNA3
pcDNA3_RhoGDIα
pcDNA3
pcDNA3_RhoGDIα

Fig.S4


Ip RhoGDIα
RhoAR68E
RhoA
RhoGDIα
RBD Pull-Down
RhoAR68E-GTP
RhoA-GTP
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RhoA
Erk1,2
vector
mRhoA
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siRhoA

Fig.S5

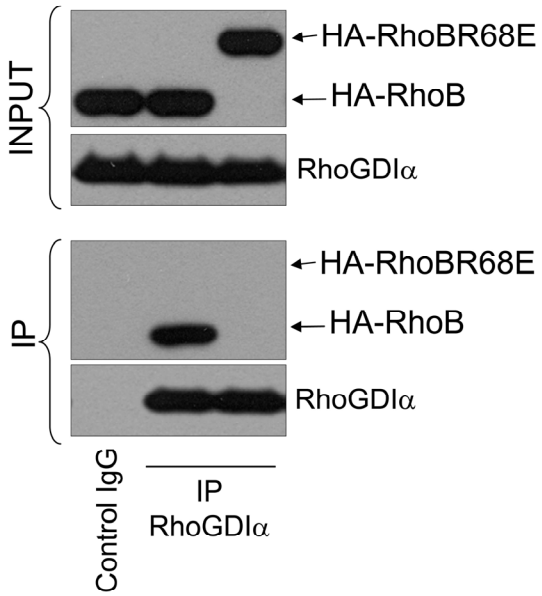
INPUT
HA-RhoBR68E
HA-RhoB
RhoGDIα
IP
HA-RhoBR68E
HA-RhoB
RhoGDIα
Control IgG
IP
RhoGDIα

Fig.S6