RhoA-GDP Regulates RhoB Protein Stability

POTENTIAL INVOLVEMENT OF RhoGDIα

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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; ROCK, 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 RhoGDIα 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-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 R210-01) and ZeocinTM (number 45-0430) were from Invitrogen. Alexa Fluor® 488 goat anti-rabbit IgG (number A21202), Alexa Fluor® 546 goat anti-mouse IgG (number A21205), and 5-ethyl-2-[4-morpholinyl]-3(2H)-benzimidazolium (Bisbenzimide H 33258—A series of 21-nucleotide long siRNAs were chemically synthesized, desalted, deprotected, and PAGE purified (Eurogentec). The 5'-GAUGUAACUGAGAAUGUGATT-3' and 5'-GAUAAAGACAGCAUGUAGGT-3' oligoribonucleotides (siRhoA) or 5'-GAUAAAGACAGCAUGUAGGT-3' and 5'-GAUAAAGACAGCAUGUAGGT-3' oligoribonucleotides (siRhoC) or 5'-AACAUUCUCAGGAAUGU-3' and 5'-AAGUUCUCCAGGAAUGU-3' oligoribonucleotides (siRho#2) were used to inhibit RhoC synthesis; the 5'-CCCACUGUGCAAACUCCU-3' and 5'-GGAGUGUG-3' oligoribonucleotides (siRac1) or 5'-GGAGUGUGUGUUG-3' or 5'-GUGUGGUUUAU-3' oligoribonucleotides were used as control (siScr) were designed by randomly mixing the sequence of the siRhoA. The 5'-AACAGUCACCGUGAUUUUUU-3' and 5'-AACAGUCACCGUGAUUUUUU-3' oligoribonucleotides were used as control for siRhoA and siRhoC. They were designed by introducing two nucleotide mismatches in the sequences of siRhoA and siRhoC, respectively. Each pair of oligoribonucleotides were 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'-GTA- GACGGGTTGGACA-3') and 5'-GGTG GGCCA-GAGGGTTGGACA-3', human mutated RhoA (mRhoA) (5'- GTACATGGGTGACCCAAAGACACG-3') and 5'-AGAGGG- CACACGTCAGCGCTGATCA-3'). For the 28 S rRNA, the efficiency of 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-STM Multilager (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
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volume of 20 \mu l containing 2 \mu l 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 \mu l 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'-GCCACAGCCGCCC-GCGCTGCA-3', reverse, 5'-CCGGCAGGGGCAGGGC-GAC-3'; and glyceraldehyde-3-phosphate dehydrogenase: forward, 5'-CTTGCGCAGGTCATCCATGACA-3', reverse, 5'-GGGATGAATTGTGCCCCAGCCTT-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 \mu g of pcDNA4/TO or pcDNA4/TO/mRhoA were transfected into cells for 20–24 h with 3 \mu l 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 \mu g/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 \mu g/ml blasticidin + 200 \mu g/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 \mu g/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 \mu g/ml blasticidin + 200 \mu g/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 (MYPYDYPDYA) 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 \mu g of plasmid was transfected into cells for 20–24 h with 3 \mu l 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 MgCl₂, 2 mm EDTA, 5 mm sodium fluoride, 0.1 mm 4-(2-aminoethyl)benzenesulfonyl fluoride, 4 \mu g/ml aprotinin, 1 mm dithiothreitol, and 0.1% Triton X-100 for 15 min on ice. The cell lysates were centrifuged at 24,000 \times g for 15 min at 4 °C. The supernatants corresponding to the cytosol fraction were collected and the pellet resuspended in ice-cold lysis buffer containing 1% Triton X-100 and centrifuged at 24,000 \times 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 \mu g/ml aprotinin. Lysates were centrifuged for 8 min at 13,000 \times 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 \mu g of GST-PBD protein containing the Cdc42 and Rac binding region of PAK-1B, or GST-RBD protein containing the Rho binding region of rhoetakin affinity linked to glutathione-Sepharose beads. The beads were washed 4 times in lysis buffer and boiled in 60 \mu l 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 \mu g/ml cycloheximide, at a concentration that effectively blocked synthesis of the RhoB protein (26),
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 (DAPI). 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 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. 4B) 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. 3A). 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. 3A). The induction of RhoB following RhoA silencing was observed at a concentration of siRNA as low as 0.2 nM (Fig. 3B). 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
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. 4A). 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. 4B). 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. 5A). 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 RhoGTPases 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. 5B). 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. 5A). The simultaneous knockdown of RhoA, RhoB, and RhoC effectively suppressed the actin stress fibers network as compared with its maintenance in

FIGURE 2. RhoA or 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 RhoB (siRhoB). Immediately after washing and trypsinization, each pool of transfected cells was separated in two halves and transfected either with empty pcDNA3 or pcDNA3/mRhoA. The strong (more than 10 times the physiological level) and transient (−24 h) re-expression of RhoA reversed at least partly the overexpression of RhoB (Fig. 4A). 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. 4B). 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).

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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 double knockdown RhoA + RhoC (Fig. 5C). 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. 6A). 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. 6B 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 P13K 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. 5B). Addition of geranylgeranylprophosphate 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. 5B). 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 P13K 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 (supplementary Fig. S3) suggesting that neither ERK1,2, p38MAPK, nor P13K 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 HS578T cells (Fig. 7A), 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 treatment a functional RhoAN19 by using a pull-down assay (Fig. 7). RhoAN19 is visible as a slower migrating band in Fig. 7. As assessed by real-time quantitative PCR measurements performed on 3 clones of PC-3/TR/control and 3 clones of PC-3/TR/mRhoA are illustrated. 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) 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 RhoA, Erk1,2, RhoB and 28s 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 treatment 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. 7B, 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. 7C). 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...
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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 48 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. 8A). Upon RhoGDIα overexpression, RhoB was mainly membrane-bound (supplementary Fig. S4B). The GTP-bound RhoB form was also increased and represented between 2 and 3% of total RhoB (supplementary Fig. S4A). This upregulation of RhoB was associated with a strong increase of its half-life (Table 1) suggesting that RhoGDIα may protect RhoB from degradation (Fig. 8B). 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. 8C). 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. 8D. 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. 8D). To further investigate the molecular mechanism driving RhoB stabilization, we tested its interaction with RhoGDIα. Coimmunoprecipitation 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 (supplementary Fig. S6). The generation of the R68E mutation in RhoB completely abolished its interaction with RhoGDIα (supplementary 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
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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

can be observed in overexpression experiments, a direct role for RhoGD1α 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

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.

TABLE 1
Wild type and mutated RhoB stability
RhoB, HA-RhoB, and HA-RhoB<sub>R68E</sub> 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_RhoGD1α         | 13.5 ± 3.5 h |
| HA-RhoB half-life      |              |
| pcDNA4_HA-RhoB + empty pcDNA3 | 2.5 ± 0.1 h |
| pcDNA4_HA-RhoB + pcDNA3_RhoGD1α | 4.6 ± 0.3 h |
| HA-RhoB<sub>R68E</sub> half-life |          |
| pcDNA4_HA-RhoB<sub>R68E</sub> + empty pcDNA3 | 4.9 ± 1.5 h |
| pcDNA4_HA-RhoB<sub>R68E</sub> + pcDNA3_RhoGD1α | 3.6 ± 1.3 h |

FIGURE 7. The regulation of RhoB is mediated by RhoA-GDP. A, HS787 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 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 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).
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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, or 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 p21CIP1 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. Laprière, B. V. Nusgens, and C. F. Deroanne, unpublished data.

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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 RhoGDIG, 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 RhoGDIA was a better candidate. Moreover, it was described as interacting with RhoB in some studies (39). Our data strongly suggest that RhoGDIA 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 RhoGDIA is involved, at least partly, in the regulation of RhoB reported here. The affinity of RhoGDIA is likely higher for RhoA and Rac than for RhoB because the latter lacks Ser-188, a residue affecting positively the binding to RhoGDIA upon phosphorylation by PKA (7, 40). In overexpression experiments, an interaction between RhoB and RhoGDIA was actually observed. Moreover, transfected RhoGDIA can stabilize wild-type RhoB and not RhoBR68E that has lost the ability to bind RhoGDIA. However, a direct stabilization of endogenous RhoB by RhoGDIA remains elusive. Alternatively, it could involve the interaction of RhoGDIA with some proteins other than RhoB. Quantitation of the RhoGDIA 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 RhoGDIA 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 RhoGDIA by RhoA silencing could also contribute to the up-regulation of the RacGTPase 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 RhoGDIA (32) and, as mentioned above, increases in parallel the RhoB protein level. Recently, Wong and co-workers (10) reported that the alteration of RhoGDIA-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 RhGTPases.

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 RhoGDIA is a necessary component for stabilizing RhoB that could act directly, indirectly, or as part of a multiprotein 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.

REFERENCES

1. Bishop, A. L., and Hall, A. (2000) Biochem. J. 348, 241–255
2. Arozarena, I., Matallanas, D., and Crespo, P. (2001) J. Biol. Chem. 276, 21878–21884
3. Meridriss-Messadi, C., Schnelzer, A., and Bokoch, G. M. (2004) Mol. Cell 15, 117–127
4. Deridriss-Messadi, C., Rocklin, G., Seo, Y. J., and Bokoch, G. M. (2006) Mol. Biol. Cell 17, 4760–4768
5. Forget, M. A., Desrosiers, R. R., Gingras, D., and Beliveau, R. (2002) Biochem. J. 361, 243–254
6. Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M., and Bertoglio, J. (1996) EMBO J. 15, 510–519
7. Rolli-Derikinderen, M., Sauzeau, V., Boyer, L., Lemeiche, E., Baron, C., Henrion, D., Loirand, G., and Pacaud, P. (2005) Circ. Res. 96, 1152–1160
8. Carol, R. J., Takeda, S., Linstead, P., Durrant, M. C., Kakesova, H., Derbyshire, P., Drea, S., Zarsky, V., and Dolan, L. (2005) Nature 438, 1013–1016
9. Di-Poi, N., Faure, J., Grizot, S., Molnar, G., Pick, E., and Dagher, M. C. (2001) Biochemistry 40, 10014–10022
10. Wong, K. W., Mohammadi, S., and Isberg, R. R. (2006) J. Biol. Chem. 281, 40579–40388
11. Ridley, A. J. (2004) Breast Cancer Res. Treat. 84, 13–19
12. Adnane, J., Muro–Cacho, C., Mathews, L., Sebti, S. M., and Munoz–Alvarez, A. (2002) Clin. Cancer Res. 8, 2225–2232
13. Mazieres, J., Tillement, V., Allal, C., Clanet, C., Bobin, L., Chen, Z., Sebti, S. M., Favre, G., and Pradines, A. (2005) Exp. Cell Res. 304, 354–364
14. Jiang, K., Sun, J., Cheng, J., Djeu, J. Y., Wei, S., and Sebti, S. (2004) Mol. Cell. Biol. 24, 5565–5576
15. Couderc, B., Pradines, A., Rafii, A., Golzio, M., Deviers, A., Allal, C., Berg, D., Penary, M., Teissie, J., and Favre, G. (2008) Cancer Gene Ther. 15, 456–464
16. Du, W., and Prendergast, G. C. (1999) Cancer Res. 59, 5492–5496
17. Liu, A., Cerniglia, G. J., Bernhard, E. J., and Prendergast, G. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6192–6197
18. Deroanne, C. F., Hamelryckx, D., Ho, T. T., Lambert, C. A., Catroux, P., Lapiere, C. M., and Nusgens, B. V. (2005) J. Cell Sci. 118, 1173–1183
19. Canguilhem, B., Pradines, A., Baudouin, C., Bovy, C., Lajoie-Mazenc, I., Charveron, M., and Favre, G. (2005) J. Biol. Chem. 280, 43257–43263
20. Zhang, B., Zhang, Y., Daigher, M. C., and Shacter, E. (2005) Cancer Res. 65, 6054–6062
21. Deroanne, C., Vouret–Craviari, V., Wang, B., and Pouyssegur, J. (2003) J. Cell Sci. 116, 1367–1376
22. Chaplet, M., Detry, C., Deroanne, C., Fisher, L. W., Castronovo, V., and Bellacche, A. (2004) Biochem. J. 384, 591–598
23. Yoshida, M., Sawada, T., Ishii, H., Gerszten, R. E., Rosenzweig, A., Gimbrone, M. A., Jr., Yasukochi, Y., and Numano, F. (2001) Arterioscler. Thromb. Vasc. Biol. 21, 1165–1171
24. Ren, X. D., Kiooses, W. B., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
25. Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999) J. Cell Biol. 147, 1009–1022
26. Stamatakis, K., Cornuda–Morollon, E., Hernandez–Perera, O., and Perez–Sala, D. (2002) J. Biol. Chem. 277, 49389–49396
27. Simpson, K. J., Dugan, A. S., and Mercurio, A. M. (2004) Cancer Res. 64, 8694–8701
28. Pulverer, B. (2003) Nat. Cell Biol. 5, 489–490
29. Fritz, G., and Kaina, B. (1997) J. Biol. Chem. 272, 30637–30644

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