RhoA regulates RhoB through a RhoGDIα-dependent mechanism.

T.T.Giang Ho, Sofia D. Merajver°, Charles M. Lapière, Betty V. Nusgens and Christophe F. Deroanne§

Laboratory of Connective Tissues Biology, University of Liège, Tour de Pathologie, B23/3, B-4000 Sart Tilman, Belgium

and

° Breast and Ovarian Cancer Risk Evaluation Program, University of Michigan Comprehensive Cancer Center, 7217 CCGC

1500 East Medical Center Drive

Ann Arbor, MI 48109-0948, USA

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ABBREVIATIONS: Erk, extracellular signal-regulated kinase; HSF, Human Skin Fibroblast; p38MAPK, p38 Mitogen-Activated Kinase; PI3K, phosphatidylinositol 3’-OH kinase; ROCK, Rho kinase.

§ To whom correspondence should be addressed at: Laboratory of Connective Tissues Biology, University of Liège, Tour de Pathologie, B23/3, B-4000 Sart Tilman, Belgium. Tel.: +32 (0)4 366 24 59; Fax: +32 (0)4 366 24 57; Email: c.deroanne@ulg.ac.be
SUMMARY

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 sub-class, 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 level that was rescued by re-expressing RhoA and related to an enhanced half-life of the protein. The stabilization of RhoB was not dependent on isoprenoid biosynthesis, ROCK, Erk1,2, p38MAPK or PI3K pathways but required RhoGDIα. The forced expression of RhoGDIα increased RhoB half-life while its knock-down antagonized the induction of RhoB following RhoA silencing suggesting that, in basal conditions, RhoGDIα is rate-limiting and the suppression of RhoA makes it available to stabilize RhoB. The competition for binding to RhoGDIα was not limited to members of the Rho sub-class. Their progressive depletion with two different sets of siRNAs simultaneously increased the binding of Rac1 to RhoGDIα and decreased its activation level while the regulation of Rac1 activity was abolished by silencing RhoGDIα. Our results highlight RhoGDIα-dependent cross-talks which regulate both stability and activity of RhoGTPases.
INTRODUCTION

The small GTPases of the Rho family are at the crossroads of signalling pathways initiated by receptors to diffusible biological mediators and those depending on cell-adhesion receptors. They are key signalling molecules regulating a plethora of biological pathways (Bishop and Hall, 2000). The RhoGTPases 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 GEFs that catalyze the exchange of GDP to GTP, the GAPs which increase the intrinsic GTPase activity of the RhoGTPase and the RhoGDIs that inhibit the exchange of GDP to GTP. RhoGDI constitutes a family with three mammalian members: RhoGDIα, the ubiquitously expressed archetypal member of the family; Ly/D4-GDI or RhoGDIβ, which has haematopoietic tissue-specific expression; and RhoGDI-3 or -γ, which is membrane-anchored and is preferentially expressed in brain, pancreas, lung, kidney and testis. RhoGDIs are usually perceived as “static” inhibitors preventing the activation by the RhoGTPases of their downstream effectors. Accumulating evidence suggests that RhoGTPase-RhoGDI complexes are highly dynamics. Phosphorylation of RhoGDIα by various kinases which decreases its affinity for RhoGTPases is one likely mechanism used by receptors to activate specific RhoGTPases (DerMardirossian et al., 2004; DerMardirossian et al., 2006). By contrast, phosphorylation of the RhoGTPases themselves generally seems to increase their affinity for RhoGDIα thus leading to signal termination (Forget et al., 2002; Lang et al., 1996; Rolli-Derkinderen et al., 2005). In addition, a more complex role has been proposed 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 (Carol et al., 2005; Di-Poi et al., 2001). 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 (Wong et al., 2006).
Among the RhoGTPase family, the Rho sub-class includes RhoA, its closely related homolog RhoC, and RhoB. RhoB by contrast to RhoA and RhoC is a short-lived protein displaying anti-tumorigenic function (Ridley, 2004). In various solid tumors, RhoB expression diminished in parallel with tumor progression (Adnane et al., 2002; Mazieres et al., 2004). Its overexpression antagonizes cell migration and metastasis (Jiang et al., 2004). 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 (Du and Prendergast, 1999; Liu et al., 2001). 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 highlight the regulation operated by Cdc42 on MMP-1 (Deroanne et al., 2005) has several advantages as compared to classical methods. Beside a higher specificity, siRNA suppresses both GDP- and GTP-bound forms which better allows to evaluate the contribution of RhoGTPase-RhoGDI complex in the global function of each specific RhoGTPase. Silencing RhoA led to an increase of RhoB by a post-transcriptional mechanism extending the half-life of the protein. Investigation of various intracellular signalling pathways demonstrated a major role of a RhoGDIα-dependent mechanism in this process. Our data also suggest that RhoGDI-dependent regulations are likely widespread and could be responsible for numerous cross-talks between RhoGTPases.
RESULTS

Morphological suggestion of the activity of RhoB

The key role of RhoA in the organization of actin cytoskeleton and the regulation of cell shape has been clearly established. However, its specific repression -up to 95%- in various cell types with two different sets of siRNA did not alter cell morphology neither actin stress fibers formation (Deroanne et al., 2003; Deroanne et al., 2005 and results not shown). According to the data recently published by Simpson and co-workers (Simpson et al., 2004), this lack of morphological alteration could be due to a compensatory mechanism between RhoA and RhoC. We tested that proposition by the double silencing of RhoA and RhoC. Although the morphology of human breast adenocarcinoma cell HS578T was altered by this double knock-down, stress fibers were still visible, suggesting that a residual “Rho-like” activity was still present (Fig.1). This might be related to the activity of the third member of the Rho sub-class, RhoB. This RhoGTPase is also able to activate effectors like ROCK and induce the formation of actin stress fibers.

RhoA-dependent regulation of RhoB

RhoB is a short-lived protein and in basal conditions its level is very low as observed by Western-blot analysis of whole-cell HS578T lysate. It was also barely detectable in mock-transfected cells or in cells transfected with a control siRNA (Fig.2). The knockdown of RhoA in HS578T cells induced a significant increase of RhoB protein level (Fig.2). RhoB concentration was also slightly increased upon RhoC silencing while the double silencing of RhoA and RhoC induced its dramatic increase as compared to the single silencing of RhoA (Fig.2). The negative regulation of RhoB operated by RhoA and RhoC was also observed in human fibrosarcoma cells HT1080, melanoma cells A2058, in primary human skin fibroblasts (HSF) (Fig.2) and in human prostate adenocarcinoma cells PC-3 (Fig.4B). These observations
suggest that this is a wide-spread mechanism. Similar results were observed by using a second siRNA targeting another sequence of RhoA or RhoC mRNA while neither Rac1 nor Cdc42 silencing affected RhoB protein level (Fig.3A). The induction of RhoB following RhoA silencing was observed at concentration of siRNA as low as 0.2 nM (Fig.3B). To 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 neutral mutations impairing the silencing by this siRNA (mRhoA). HS578T cells were first transfected with the scrambled siRNA or with the first siRNA targeting RhoA. Immediately after washing, each pool of transfected cells was trypsiniized, separated in two halves and transfected either with empty pcDNA3 or pcDNA3/mRhoA as described in Materials and Methods. The transient re-expression of RhoA reversed at least partly the overexpression of RhoB (Fig.4A). The remaining 40% of RhoB could be due to the presence of some cells that have not been transfected by pcDNA3/mRhoA. The strong (more then 10 times the physiological level) and transient (~24 hours) re-expression of RhoA is obviously not the best way 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 of scrambled siRNA or 6 nM of siRhoA. Immediately after washing, each pool of transfected cells was trypsiniized, separated in two halves and cultured for 2 days either in absence or in presence of 1 µg/ml tetracycline. Cells were then processed for Western blot or RT-PCR analysis. RT-PCR analysis revealed that endogenous RhoA was similarly repressed in absence or presence of tetracycline while
the mutated RhoA was significantly induced by tetracycline. The re-expression of the RhoA protein in these conditions nearly completely reversed the over-expression of RhoB while the 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 (Fig.4B).

*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 was paralleled by an enhanced level of RhoB active form (Fig.5A). However, an efficient activation of the downstream effectors also requires membrane association of the RhoGTPase (del Pozo et al., 2000). To determine the sub-cellular localization of RhoB, a differential extraction procedure was used which revealed that the up-regulated RhoB is mainly associated to the membrane fraction (Fig.5B). These observations suggested that RhoB could be functional and therefore responsible for the residual “Rho-like” activity observed in figure 1. To address its role in the persistence of actin stress fibers following RhoA and RhoC silencing, a simultaneous repression of RhoA, RhoC and RhoB was performed in HS578T cells. Western blot analysis and pull-down assays collected 48h after transfection revealed the efficient repression of total and active RhoB (Fig.5A). The simultaneous knockdown of RhoA, RhoB and RhoC completely suppressed the actin stress fibers network as compared to the double knockdown RhoA+RhoC which induced the expression of a RhoB that is functional and able to regulate cytoskeletal organization (Fig.5C).
RhoB up-regulation is related to a prolonged half-life of the protein.

As assessed by RT-PCR measurements following RhoA or RhoC silencing, the induction of RhoB was not related to an increase of its mRNA level in primary human skin fibroblasts (HSF) and HS578T cells (Fig.6A). It was clearly up-regulated by the double silencing RhoA+RhoC in both cell types. The stability of the RhoB protein was determined in HS578T cells by blocking protein synthesis with cycloheximide. The half-life of RhoB was of 2 hours in cells transfected with the scrambled siRNA, of 4 hours in RhoC-silenced cells and greater than 8 hours in RhoA-silenced cells. The double silencing of RhoA and RhoC similarly enhanced the half-life of RhoB (Fig.6B) suggesting that in this condition the RhoB protein level was up-regulated by both transcriptional and post-translational mechanisms. Similar observations were also made in HSF (not shown).

The induction of RhoB is not dependent on isoprenoid biosynthesis, ROCK, Erk1,2, p38MAPK or PI3K pathways.

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 the translocation to the membrane (Stamatakis et al., 2002). It was also observed in our models (Figure 5B). Addition of geranylgeranyl-pyrophosphate and/or farnesyl-pyrophosphate rescued the simvastatin-induced up-regulation of RhoB (Stamatakis et al., 2002). A similar procedure used in RhoA and/or RhoC silenced cells did not alter the up-regulation of RhoB (not shown). Moreover, by using a differential extraction procedure, we observed that RhoB up-regulated by silencing RhoA was associated with the membrane fraction while the RhoB induced under simvastatin treatment was cytoplasmic (Fig.5B). These results rule out the implication of isoprenoid biosynthesis 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 like ROCK. To test this hypothesis, ROCK was specifically inhibited in HS578T cells with Y-27632 during 24 or 48 hours of culture. This treatment barely modulated RhoB protein level in HS578T cells (not shown). Furthermore, inhibition of either Erk1,2, p38MAPK or PI3K pathways with pharmacological inhibitors did not alter the RhoA-dependent regulation of RhoB (not shown).

The induction of RhoB is RhoGDIα-dependent.

Several studies suggested that in addition to its role in regulating RhoGTPase activation, RhoGDIs protect some RhoGTPases from degradation (Rolli-Derkinderen et al., 2005). To address the role of RhoGDIα in RhoB protein stability we first transfected HS578T cells with an expression vector encoding the whole coding sequence of human RhoGDIα. Its forced expression strongly enhanced RhoB protein level as compared to cells transfected with an empty vector where RhoB was barely detectable (Fig. 7A). This up-regulation of RhoB was associated with a strong increase of its half-life, 2 hours in control conditions as compared to more than 8 hours when RhoGDIα is overexpressed, suggesting that RhoGDIα could protect RhoB from degradation (Fig. 7B). This potential role was tested by performing simultaneous knockdown of RhoA and RhoGDIα in HS578T cells. The level of RhoB protein 48 hours following transfection with the two different siRNA targeting RhoA alone was reduced by more than 70% if RhoGDIα was simultaneously knocked-down (Fig. 7C). These results support the role of RhoGDIα as a significant mediator of the RhoA-dependent regulation of RhoB.

The progressive depletion of Rho-subclass members inhibited Rac1 activity.

From these results, it could be expected that such RhoGDIα-dependent regulations are not limited to the Rho sub-class. By mean of co-immunoprecipitation experiments with lysates of
HS578T cells 48 hours after transfection, we observed that the double silencing of RhoA+RhoC, with two different sets of siRNA, increased strongly the association between RhoGDI\(\alpha\) and Rac1 (Fig. 8A). These results prompted us to analyse the activation level of Rac1 in the same experimental conditions by using a pull-down assay. We observed that the simultaneous repression of RhoA and RhoC did not affect the expression level of Rac1 but reduced its activation level by 70-90\% (Fig. 8B). The repression of Rac1 activity was even more pronounced (over 90\%) upon simultaneous knockdown of RhoA, RhoC and RhoB (Fig. 8C). The simultaneous knockdown of RhoGDI\(\alpha\) completely abrogated these regulations of Rac1 activity demonstrating that they are RhoGDI\(\alpha\)-dependent (Fig. 8B and C). Altogether, these results demonstrate that RhoGDI\(\alpha\) plays a critical role in the cross-talks regulating the stability and/or the activity of RhoGTPases.
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 common 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 definitively validate our results. The transient rescue of RhoA silencing effectively repressed the RhoB up-regulation but a residual level of RhoB was still present and probably related to technical limitation of the procedure: (i) a variable proportion of the cells (10~30%) may not be transfected by the mRhoA expression vector; (ii) the target-gene is too strongly re-expressed (up to 10 times the physiological level) and (iii) this re-expression is more transient than the silencing (24 hours compared to several days). To circumvent these problems, we generated clones of PC-3 cells expressing the mRhoA in a tetracycline-dependent way. The concentration of tetracycline used here (1µg/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 thus definitively confirming the specificity of the negative regulation operated by RhoA on RhoB. This technology also opens the way to in vivo rescue experiments by feeding animals - in which these cells could be injected to evaluate, for instance, their tumorigenic potential- with stable tetracycline analog like doxycycline.
No modulation of RhoB mRNA level in RhoA-silenced cells was observed by RTPCR 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 (Ho, et al. unpublished data). The increase of RhoB occurs at a post-transcriptional level through a stabilisation of the protein as assessed by half-life measurements. Fritz and Kaina (Fritz and Kaina, 1997) reported the regulation of the RhoB gene by overexpressing the dominant-negative mutant RhoAN19. The results reported here demonstrate that the silencing of a RhoGTPase does not simply mimic the overexpression of a dominant-negative mutant which targets guanine-nucleotide exchange factors (GEFs). While some Rho GEFs are highly specific, other activate multiple Rho GTPases (Schmidt and Hall, 2002). 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 GEF(s) which 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. Such a regulation is only visible following depletion of the active and inactive pool of RhoA suggesting that both forms could be involved in RhoB destabilisation. Using a similar approach, Simpson et al. (Simpson et al., 2004) recently reported a cross-regulation between RhoA and RhoC. Thus, our results suggest that such regulations, uncovered by our siRNA-based approach and undetectable by conventional tools, are widespread in the RhoGTPase family.

By contrast to RhoA and RhoC reported to be up-regulated in various cancer, RhoB displays properties that might participate in tumor suppression (Huang and Prendergast, 2006). As assessed by pull-down assay and indicated by the presence of actin stress fibers the up-
regulated RhoB is indeed biologically active. It could contribute to the anti-tumoral effect of siRNA targeting RhoA or RhoC reported recently in vitro and in vivo (Pille et al., 2005). Furthermore, we observed that the induction of the cell cycle inhibitor p21/CIP1 following RhoC silencing in PC-3 cells is RhoB-dependent (Ho et al., unpublished data).

It is well documented that alterations of post-translational prenylation of RhoB by inhibitors of the mevalonate pathways or of farnesyl transferase can increase the expression of RhoB by acting at a transcriptional level (Delarue et al., 2007) but also through a modulation of the protein stability (Stamatakis et al., 2002). However, the RhoB protein induced by treatment with one of these inhibitors, simvastatin, as we showed here is cytosolic while the RhoB induced by repressing RhoA like the RhoB expressed in basal conditions, is associated with the membrane fraction. This suggests that the silencing of RhoA did not affect post-translational lipid modifications required for RhoB sub-cellular localization. Moreover, the increased half-life of RhoB following RhoA silencing was not reversed by supplementation of prenylation precursors like 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 (Stamatakis et al., 2002). In physiological conditions, the isoprenyl moiety is masked upon binding to RhoGDIIs (Hoffman et al., 2000) thus explaining, at least in part, how RhoGDI can protect the RhoGTPases from ubiquitin-mediated degradation as reported for RhoA (Rolli-Derkinderen et al., 2005). Our data clearly demonstrated that RhoGDIα can stabilize RhoB and is operational in the up-regulation of RhoB following RhoA silencing. The affinity of RhoGDIα is likely higher for RhoA and RhoC than for RhoB since the latter lacks Ser188, a residue affecting positively the binding to RhoGDIα upon phosphorylation by PKA (Dovas and Couchman, 2005; Rolli-Derkinderen et al., 2005). RhoGDIα forms 1:1 complexes with the RhoGTPases. Quantitation of RhoGDIα level in various cell types has revealed that
its molar amount is roughly equal to the molar amount of the three GTPases RhoA, Rac1 and Cdc42 (Michaelson et al., 2001). In physiological conditions RhoGDIα is thus likely rate-limiting and its over-expression 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 RhoC protein level that we and others observed upon RhoA depletion (Fig.2) and (Simpson et al., 2004). The progressive silencing of the Rho-subclass members by co-transfection of several siRNAs increased gradually the proportion of RhoGDIα available for binding to other RhoGTPases like Rac1 thus leading to the indirect regulation of its activity as reported in this study. Recently, Wong and co-workers reported that the alteration of RhoGDI-dependent cross-talk between RhoA and Rac1 suppresses integrin-mediated bacterial uptake (Wong et al., 2006). Altogether these data suggest that such interplays should be a widespread mechanism to control the stability and the activity of the RhoGTPase family.

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. It could be also related to the inability of the mutated forms of RhoGTPase to bind RhoGDIα (Michaelson et al., 2001; Strassheim et al., 2000) which render these tools inappropriate to uncover RhoGDI-dependent regulations. Our results extend the role of RhoGDI, making it a key intermediate in the coordination of RhoGTPase action by regulating not only their activation but also their stability. 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.
MATERIALS AND METHODS

Reagents and cells

Rabbit anti-RhoC was previously described (Kleer et al., 2002). The other antibodies were purchased from the following manufacturers: mouse anti-RhoA (#sc-418), rabbit anti-RhoB (#sc-180), rabbit anti-RhoGD1α (#sc-360) were from Santa Cruz Biotechnology; mouse anti-Rac1 (23A8) from Upstate Biotechnology; mouse anti-Cdc42 from BD Biosciences; rabbit anti-ERK-1,2 (#M-5670) was from Sigma; rabbit polyclonal anti-Tet-repressor antibody was from MoBiTec (#TET01). Y-27632 was kindly supplied by A. Yoshimura (Welfide Co, Japan), U0126 was from Calbiochem, LY294 from Sigma and SB203580 from Alexis corporation. DAPI (Bis-benzimide H 33258 #382061) was from Calbiochem. 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% FBS (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% FBS and trypsinized 1:10 every week. Human prostate adenocarcinoma cells PC-3 were amplified in F-12 Kaighn’s medium supplemented with 7% FBS.

siRNA transfection

A series of 21-nucleotides long siRNAs were chemically synthesized, desalted, deprotected and PAGE purified (Eurogentec). The 5’-GAAGUCAAGCAUUUCUGUCTT-3’ and 5’-GACAGAAAUGCUUGACUUCTT-3’ oligoribonucleotides (siRhoA) or 5’-GAAGGAUCUUCGGAUUGAUTT-3’ and 5’-AUCAUUCCGAAGAUCCUUCTT-3’ oligoribonucleotides (siRhoA#2) were used to inhibit RhoA synthesis; the 5’-
oligoribonucleotides (siRhoC) or the 5’-UCCACUUCGAGAAUGUUTT-3’ and 5’-
AACAUUCGAGAAGUGGATT-3’ oligoribonucleotides (siRhoC#2) were used to inhibit
RhoC synthesis; the 5’-CACCACUGUCCCAACACUUCTT-3’ and 5’-
GAGUGUUGGGACAGUGGUUGTT-3’ oligoribonucleotides (siRac1) or 5’-
GAUAAAGACACGAUCGAGATT-3’ and 5’- UCUCGAUCGUGUCUUAUUCTT-3’
oligoribonucleotides (siRac1#2) were used to inhibit Rac1 synthesis. The siRNAs used to
inhibit RhoB, Cdc42 or RhoGDiα synthesis were described elsewhere (Canguilhem et al.,
2005; Deroanne et al., 2005; Zhang et al., 2005). The 5’-UUGCAUACAGGACGUUATT-
3’ and 5’-UAACGAGUCCUGUAUGCAATT-3’ oligoribonucleotides used as control (siScr)
were designed by randomly mixing the sequence of the siRhoA. Each pair of
oligoribonucleotides was annealed at a concentration of 20 µM in 50 mM NaCl, 1mM EDTA,
10 mM Tris-HCl pH 7.5. SiRNA transfection was carried out as previously described
(Deroanne et al., 2003). Briefly, calcium phosphate-mediated transfection was performed
overnight (14-16 hours) on subconfluent cells at a final concentration of siRNA ranging from
0.2 nM 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 hours 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
(Perkin Elmer) with pairs of primers amplifying mRNA coding for human RhoA (5’-
GTACATGGAGTGTTCAGCAAAAGACC-3’ and 5’-GGTGGGCCAGACGGGTTGGACA-
3’), human mutated RhoA (mRhoA) (5’- GTACATGGAGTGTTCAGCAAAAGACC-3’ and
5’-AGAAGGCACAGTCGAGGCTGATCA-3’), human RhoB (5’-GTGCCTGCTGATCGTGTTCAGTAA-3’ and 5’-CTCATAGCACCTTGCAGCAGTTGA-3’), and 28S rRNA (5’-GTTCACCCACTAATAGGGAACGTGA-3’ and 5’-GGATTCTGACTTAGAGGCGTTCAGT-3’). For the 28S 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 (Deroanne et al., 2005). Briefly, 10 ng total RNA and a known copy number of the standard synthetic RNA were reverse transcribed (70°C for 15 minutes). Then, RNA-DNA heteroduplexes were denatured for 2 minutes at 94°C and amplification was carried out for 22 cycles (RhoA and mRhoA), for 28 cycles (RhoB) or for 17 cycles (28S rRNA) at 94°C for 15 seconds, 66°C for 20 seconds and 72°C for 20 seconds (10 seconds for 28S 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 Laboratories, Life Science).

Constructs for rescue of RhoA knock-down and RhoGDIα overexpression

The entire coding sequence of human RhoA was amplified by RT-PCR. The amplification product was mutated by mean of a PCR-based approach with mutated primers. Five silent mutations were introduced in the sequence targeted by the 1st siRhoA in order 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 (Deroanne et al., 2005). Briefly, cells were first transfected with 6nM of siScr or 6nM siRhoA for 14-16h following the protocol described above. Immediately after washing, each pool of cells was trypsinized and seeded in two wells of 6 wells plates. Three
hours later, 1µg of pcDNA4/TO or 1µg of pcDNA4/TO/mRhoA were transfected into cells for 20-24 hours with 3 µl of Genejuice™ (Novagen). Cells were washed and cultured for a further 24 hours 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 1µg/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 1µg/ml blasticidin+200µg/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™. 24h later, the cells were transfected with 6nM siScr or 6nM siRhoA for 14-16h 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 1µg/ml tetracycline. 48 hours 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 RhoGDIα was amplified by RT-PCR and cloned into pcDNA3 (pcDNA3_RhoGDIα). The integrity of the cDNA was confirmed by sequencing. To overexpress RhoGDIα, HS578T cells were seeded in 6 wells plates. 3 hours after seeding, 1µg of pcDNA3_RhoGDIα was transfected into cells for 20-24 hours with 3 µl of Genejuice™ (Novagen). Control cells were transfected in the same conditions with 1 µg of pcDNA3.
**Immunoprecipitation and immunoblotting**

Sub-confluent HS578T were rinsed twice with ice-cold PBS and lysed in buffer containing 0.5% Nonidet NP-40, 50mM Tris/HCl pH 7.4, 50mM NaCl, 1mM MgCl₂, 50mM NaF, 10% glycerol, 0.1mM AEBSF and 4µg/ml aprotinin. Lysates were centrifuged for 5 minutes at 16,000 g. Supernatants were incubated with 2µg rabbit anti-RhoGDIα. After 2 hours incubation at 4°C, 40 µl of protein-A-Sepharose beads were added for 1 hour to capture immune complex. Beads were washed 4 times in ice-cold lysis buffer and boiled in 80 µl SDS-PAGE lysis buffer. For immunoblotting, samples were separated on a 15% gel under reducing conditions, transferred to Immobilon-P PVDF membranes and immunodetected with the indicated antibodies. The bands were visualised with the ECL system (Amersham).

**Rho translocation assay**

A Rho translocation assay was performed as described previously (Chaplet et al., 2004; Yoshida et al., 2001). HS578T cells were incubated with lysis buffer containing 50mM Hepes pH 7.4, 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 5 mM NaF, 0.1 mM AEBSF, 4 µg/ml aprotinin, 1 mM dithiothreitol and 0.1% Triton X-100 for 5 minutes on ice. The cell lysates were centrifuged at 24,000 g for 15 minutes 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 minutes at 4°C. The supernatant corresponding to the membrane fraction was collected.

**GTPase assays**

The assay was carried out as previously described (Ren et al., 1999; Sander et al., 1999). Briefly, cells were chilled on ice and lysed in ice-cold buffer containing 1% Triton X-100, 25
mM HEPES pH 7.3, 150 mM NaCl, 4% glycerol, 0.1 mM AEBSF, 4 µg/ml aprotinin. Lysates were centrifuged for 8 min. at 13000g. Supernatants were immediately frozen in liquid nitrogen and stored at −80° until used. 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 60µl SDS-PAGE lysis buffer.

_Determination of RhoB protein stability_

48 hours after transfection with siRNA or 24 hours after transfection with plasmids (pcDNA3 or pcDNA3_RhoGDIα) cells were incubated with 20 µg/ml cycloheximide, a concentration that effectively blocked synthesis of the RhoB protein (Stamatakis et al., 2002), and the RhoB protein levels analyzed by Western blot at various time points after cycloheximide addition.

_Cytoskeleton labelling_

HS578T cells cultured for 24 hours were fixed with 3% paraformaldehyde in PBS for 15 minutes and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. The samples were blocked with 0.2% BSA in PBS for 30 minutes and incubated with 50 ng/ml of phalloidin-TRITC, to label the fibrillar actin cytoskeleton, and with 50 ng/ml of DAPI for 30 minutes. Fluorescence was analyzed with a Leica DMRB microscope.
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REFERENCES:


FIGURE LEGENDS:

Fig.1. Simultaneous knockdown of RhoA and RhoC did not completely suppress actin stress fibers.
Immediately after transfection with 40 nM of an irrelevant siRNA (siScr) or 20 nM siRhoA + 20 nM siRhoC (siRhoA+C), HS578T cells were seeded on tissue culture dishes. 48 hours later, cells were analysed by immunofluorescence labelling with phalloidin-TRITC and DAPI. Bar, 20 µm.

Fig.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 hours post-transfection, cells were lysed and analyzed by immunoblotting with specific antibodies to RhoA, RhoB, RhoC and Erk1,2.

Fig.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 hours after transfection with calcium phosphate alone (CaP), with 20 nM of an irrelevant siRNA (siScr) or with 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). The factor of induction (F.I.) was calculated from densitometric measurements of RhoB signal normalized to Erk1,2 loading compared with the same measurements in the
lysates of HS578T cells transfected with siScr. (B) Representative Western blot analysis of HS578T cells 48 hours after transfection with the indicated concentrations of siRNA. The factor of induction (F.I.) was calculated from densitometric measurements of RhoB signal normalized to Erk1,2 loading measured compared with the same measurements in the lysates of HS578T cells transfected with 2 nM of siScr.

**Fig.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 lower 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 (-tet) with 1 µg/ml of tetracycline for 48 hours and processed for RT-PCR analysis (top panel) or Western blot (middle panel). Representative analyses out 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.

**Fig.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 analysis 48 hours 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 of siScr (siScr[3X]), with 20 nM of siRhoA + 20 nM of siRhoC + 20 nM of siScr (siRhoA+C) or 20 nM of siRhoA + 20 nM of siRhoC + 20 nM of 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 DMSO alone (vehicle) were obtained as described in Experimental Procedures. Both fractions (10 µg) were analyzed by immunobloting with specific antibodies against RhoB and Erk1,2.

(C) Immediately, after transfection with either 60 nM siScr (siScr[3X]), with 20 nM of siRhoA + 20 nM of siRhoC + 20 nM of siScr (siRhoA+C) or with 20 nM of siRhoA + 20 nM of siRhoC + 20 nM of siRhoB (siRhoA+C+B), HS578T cells were seeded on tissue culture dishes for 24 hours and analysed by immunofluorescence labelling phalloidin-TRITC and with DAPI. Bar, 20 µm.

Fig.6. The up-regulation of RhoB by silencing RhoA or RhoC was related to an increase half-life of the protein.

(A) RT-PCR analysis of RhoB mRNA level and 28S rRNA was perform with total RNA extracted from HSF or HS578T cells 48 hours after transfection with an irrelevant siRNA at 20 or 40nM (siScr or siScr[2X]), with 20 nM of the first or the second siRNA targeting RhoA (siRhoA or siRhoA#2), with 20 nM of the first or the second siRNA targeting RhoC (siRhoC or siRhoC#2) or with 20 nM of the first or the second siRNA targeting RhoA + 20 nM of the first or the second siRNA targeting RhoC (siRhoA+C or siRhoA#2+C#2). Representative analyses of 3 independent experiments are shown.

(B) HS578T cells were transfected with 20 or 40 nM of an irrelevant siRNA (siScr or siScr[2X]), with 20nM of the first siRNA targeting RhoA (siRhoA) or RhoC (siRhoC), or with 20nM of siRhoA + 20nM siRhoC (siRhoA+C). Forty eight hours after transfection, cells were supplemented with 20µg/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. It has to be noted that blots were loaded with 30µg (siScr and siScr[2X]), with 10µg (siRhoC) or 5µg of proteins (siRhoA and siRhoA+C) to obtained initial similar signal intensities.

**Fig.7. RhoB up-regulation depends on RhoGDIα availability.** (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 hours 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 hours after transfection, cells were cultured with 20µg/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 30µg (pcDNA3) or 5 ug (pcDNA3_RhoGDIα) to obtained similar initial signal intensities. (C) HS578T cells were transfected with calcium phosphate alone (CaP), with 20 nM of an irrelevant siRNA (siScr), with 20nM of the first or of the second siRNA targeting RhoA (siRhoA or siRhoA#2) alone or in combination with 20nM of siRhoGDIα (+siRhoGDIα). 48 hours after transfection, cells were processed for Western blot analysis with specific antibodies to RhoB, RhoA, RhoGDIα and Erk1,2.

**Fig.8. Progressive depletion of Rho sub-class members decreases Rac1 activity in a RhoGDIα-dependent way.** (A) RhoGDIα was immunoprecipitated (IP) with specific antibodies from lysates of HS578T cells 48 hours after transfection with calcium phosphate
alone (CaP), with 40 nM of an irrelevant siRNA (siScr[2X]), or with 20 nM of the first or the second siRNA targeting RhoA + 20 nM of the first or the second siRNA targeting RhoC (A+C or A#2+C#2). Rac1 associated with RhoGDIα was measured by Western blot analysis of the IP with specific antibodies. The fold induction (F.I.) is calculated from densitometric measurements of Rac1 co-immunoprecipitated with RhoGDIα normalized to the Rac1 measured in whole lysates before immunoprecipitation (INPUT) in the various conditions compared with the same measurements in the lysates of HS578T cells transfected with calcium phosphate alone taken as 1.0. Representative results of three independent experiments are shown.

(B-C) Rac1 activity is determined by the amount of GST-PBD-bound Rac1 (Rac1-GTP) normalized to total Rac1 in whole cell lysates of HS578T 48h after transfection with 40 or 60 nM of an irrelevant siRNA (siScr[2X] or siScr[3X]) or with various combinations of 20nM of the first or the second siRNA targeting RhoA (A or A#2), RhoC (C or C#2) or RhoB (B or B#2). In the three right-hand lanes, the transfection mix were supplemented with 20 nM of the siRNA targeting RhoGDIα (+siRhoGDIα).
Fig. 1

Phalloidin

DAPI

siScr  siRhoA+C
Fig. 2

- HS578T
  - RhoB
  - RhoA
  - RhoC
  - Erk 1,2

- A2058
  - RhoB
  - Erk 1,2

- HT1080
  - RhoB
  - Erk 1,2

- HSF
  - RhoB
  - Erk 1,2

(siSc, siRhoA, siRhoC, siScr [2X], siRhoA+C)
Fig. 3
A

Western blots

RhoA
Erk 1,2
RhoB

vector
mRhoA
vector
mRhoA

siScr
siRhoA

RhoB (A.U.)

vector
mRhoA
vector
mRhoA

B

RT-PCR

PC-3/TR/mRhoA

RhoA
mRhoA

* 28s

siScr
siRhoA

Western blots

RhoA
Erk 1,2
RhoB

+tet

PC-3/TR/mRhoA

Fig.4
**Fig. 5**

A. Western blot analysis showing RhoB-GTP, Total RhoB, and Erk 1,2 levels in different siRNA treatments: siScr, siRhoA, siRhoC, siScr[3X], siRhoA+C, siRhoA+C+B.

B. Immunoblot analysis of RhoB and Erk 1,2 in Cytosol and Membrane fractions across different treatments: siScr, siRhoA, siRhoC, siScr[2X], Simvastatin, vehicle.

C. Fluorescence microscopy images stained with Phalloidin and DAPI, depicting the effect of different siRNA treatments: siScr[3X], siRhoA+C, siRhoA+C+B.
Fig. 6

A) HS578T and HSF cells were transfected with siRNA targeting RhoA, RhoC, or both. Western blot analysis was performed to compare RhoB expression levels in the presence or absence of cycloheximide.

B) The effect of siRNA on RhoB expression was further analyzed by Western blotting. The graph shows the decrease in RhoB levels over 8 hours of treatment with different siRNA combinations.
Fig. 7
**Fig. 8**

A. IP RhoGDIα

- **Input**
  - **F.I.**:
    - CaP: 1.0
    - siScr[2X]: 1.2
    - A+C: 3.4
    - A+C#2: 5.4

- **IP RhoGDIα**
  - **Rac1**

B. Rac1-GTP

- **F.I.**:
  - siScr[2X]: 1.0
  - A+C: 0.3
  - A+C#2: 0.1
  - siScr[3X]: 1.3
  - A+C#2: 2.0
  - siScr[3X] + siRhoGDIα: 2.5

- **Total Rac1**

- **RhoGDIα**

C. Rac1-GTP

- **F.I.**:
  - siScr[3X]: 1.0
  - A+C+B: 0.08
  - A+C#2+B#2: 0.03
  - siScr[3X] + siRhoGDIα: 2.1
  - A+C+B: 1.9
  - A+C#2+B#2: 1.9

- **Total Rac1**

- **RhoGDIα**