Biol. Chem., Vol. 387, pp. 311–317, March 2006 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2006.041

p190-RhoGAP as an integral component of the Tiam1/Rac1-induced downregulation of Rho

Ulrike Herbrand and Mohammad Reza Ahmadian*

Max Planck Institute of Molecular Physiology, Department of Structural Biology, Otto-Hahn-Strasse 11, D-44227 Dortmund, Germany

*Corresponding author
e-mail: reza.ahmadian@mpi-dortmund.mpg.de

Abstract

The Rho family of small GTPases plays a central role in intracellular signal transduction, particularly in reorganization of the actin cytoskeleton. Rho activity induces cell contractility, whereas Rac promotes cellular protrusion, which counteracts Rho signaling. In this regard, the reciprocal balance between these GTPases determines cell morphology and migratory behavior. Here we demonstrate that Tiam1/Rac1 signaling is able to antagonize Rho activity directly at the GTPase level in COS-7 cells. p190-RhoGAP plays a central regulatory role in this signaling pathway. Interfering with its activation by Src-kinase-dependent tyrosine phosphorylation or its recruitment to the membrane through interaction with the SH2 domains of p120-RasGAP blocks the Tiam1-mediated rapid downregulation of Rho. This process is mediated by Rac1, but not by Rac2 or Rac3 isoforms. Our data provide evidence for a biochemical pathway of the reciprocal regulation of two related small GTPases, which are key elements in cell migration.

Keywords: GTPase; p115-RhoGEF; p190-RhoGAP; PDZ-RhoGEF; Rac; Rho; Tiam1.

Introduction

The family members of Rho-like GTPases are regulators of diverse cellular signaling pathways, including cytoskeletal organization, transcription and cell cycle progression (van Aelst and D'Souza-Schorey, 1997; Etienne Manneville and Hall, 2002). As molecular switches, Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state (Vetter and Wittinghofer, 2001). This cycling is regulated by three main classes of proteins: guanine nucleotide dissociation inhibitors (GDIs) that control the partitioning of Rho GTPases between the cytosol and membrane compartments (Dovas and Couchman, 2005); guanosine nucleotide exchange factors (GEFs) that facilitate the GDP/GTP exchange, resulting in the activation of GTPases (Rossman et al., 2005); and GTPase activating proteins (GAPs) that inactivate the GTPases by stimulating their GTP-hydrolysis reaction (Scheffzek and Ahmadian, 2005). Activated GTPases such as Cdc42, Rac1 and RhoA induce the assembly and organization of actin structures including filopodia, lamellopodia and stress fibers, respectively, by binding and activating the downstream effectors (Hall, 1998; Bishop and Hall, 2000).

Several studies have demonstrated that activation of Rac1 counteracts RhoA activity (Kozma et al., 1997; Horwitz and Parsons, 1999; Sander et al., 1999; van Leeuwen et al., 1999; Zondag et al., 2000; Noren et al., 2001). The balance between Rac and Rho activity determines neuronal morphology by controlling growth cone development or growth cone collapse (Kozma et al., 1997), and controls the morphology and motile behavior of NIH3T3 fibroblasts (Sander et al., 1999). Furthermore, it has been reported for epithelial cells and NIH3T3 fibroblasts that this effect accompanies the shift from a mesenchymal to an epithelial phenotype with an increase in cell spreading and inhibition of motility (Sander et al., 1999; Zondag et al., 2000). In this scenario, the reciprocal activities of two serine/threonine effector kinases, Rho-associated kinase (ROCK) and p21-activated protein kinase 1 (PAK1), control cell contractility (Horwitz and Parsons, 1999; Ridley, 2001). Decreased RhoA and elevated Rac1 activities are also found in confluent epithelial cells when compared to subconfluent cultures (Noren et al., 2001). It has recently been shown that the downregulation of RhoA activity by Rac1 depends on the cellular redox state that controls tyrosine phosphorylation and activation of p190-RhoGAP (Nimnual et al., 2003). Further studies have shown that Rac1 activation is a result of the engagement of cell adhesion receptors that activate p190-RhoGAP, which in turn inactivates RhoA (Noren et al., 2003).

p190-RhoGAP plays an important role in regulating Rho-mediated reorganization of the actin cytoskeleton (Ridley et al., 1993; Chang et al., 1995). It was first described as a tyrosine-phosphorylated protein associated with p120-RasGAP in v-Src-transformed cells (Ellis et al., 1990; Bouton et al., 1991). It contains an N-terminal GTP-binding domain, a glucocorticoid receptor-like central domain and a C-terminal GAP domain (Settleman et al., 1992). The latter is specific for Rho, but not for the other GTPases of the Rho family (Ridley et al., 1993). Several studies have shown that p120-RasGAP interaction is required for the recruitment of p190-RhoGAP to the plasma membrane (McGlade et al., 1993; Bryant et al., 1995; Chang et al., 1995; Hu and Settleman, 1997; Roof et al., 1998). Furthermore, Src-kinase-dependent phosphorylation of tyrosine 1105 at the end of the central domain of p190-RhoGAP is important for the binding of the SH2 domains of p120-RasGAP (Chang et al., 1995; Hu and Settleman, 1997).

PDZ-RhoGEF and p115-RhoGEF belong to the subfamily of Dbl proteins, which contain a regulator of G protein signaling-like (RGS) domain at their N-terminal region (Hart et al., 1998; Fukuhara et al., 1999; Ruemenapp

et al., 1999; Longenecker et al., 2001). p115-RhoGEF is able to accelerate the GTP-hydrolysis reaction of $G_{\alpha 12}$ and $G_{\alpha 13}$ (Kozasa et al., 1998) and hence can transmit signals from the heterotrimeric G proteins $G_{\alpha 12}$ and $G_{\alpha 13}$ to Rho (Fukuhara et al., 2001). In addition, G protein binding to the RGS domain of p115-RhoGEF was shown to partially activate its GEF activity towards Rho, suggesting that the N-terminus of p115-RhoGEF may intramolecularly regulate its GEF activity (Hart et al., 1998; Wells et al., 2002). In addition, PDZ-RhoGEF contains a PDZ domain at the very N-terminus, which mediates interaction with membrane receptors including plexins and insulin-like growth factor receptors (Taya et al., 2001; Driessens et al., 2002; Perrot et al., 2002; Swiercz et al., 2002). p115- and PDZ-RhoGEFs contain a characteristic central tandem domain organization consisting of the Dbl homology (DH) and the pleckstrin homology (PH) domains, which are responsible for the Rho-specific nucleotide exchange activity of these GEFs (Hart et al., 1998; Fukuhara et al., 1999; Ruemenapp et al., 1999; Longenecker et al., 2001).

The Rac-specific GEF Tiam1 is an important regulator of Rho GTPase functions in tumor cells (Mertens et al., 2003). Tiam1 induces T-lymphoma invasion and metastasis through Rac activation, but also inhibits migration and invasion of both human renal-cell carcinoma (Habets et al., 1994; Michiels and Collard, 1999; Engers et al., 2001) and metastatic melanoma (Uhlenbrock et al., 2004). As a GEF, Tiam1 contains a DH-PH tandem that is specific for Rac proteins (Michiels et al., 1995; Bollag et al., 2000; Haeusler et al., 2003; Fiegen et al., 2004).

In this study we investigated the biochemical mechanism by which RhoA becomes inactivated by Tiam1/Rac1 activity. We show that p190-RhoGAP regulates the signaling pathway for the Rac-induced inactivation of Rho. Its phosphorylation and interaction with p120-RasGAP, thereby forming an active heterodimer, is required to rapidly downregulate Rho by stimulating its GTP-hydrolysis reaction.

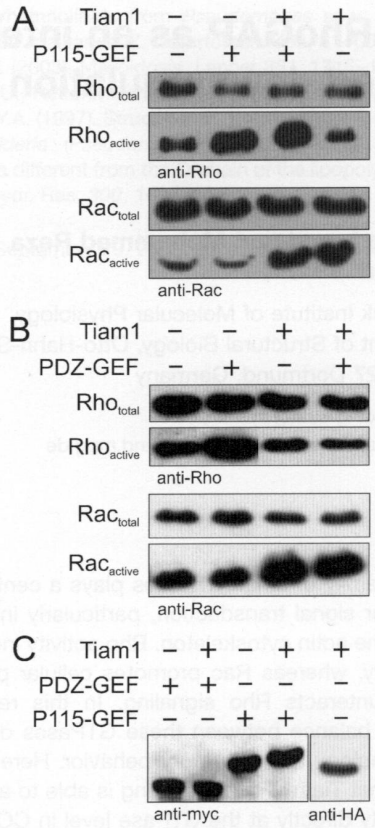

Figure 1 Tiam1-induced inactivation of Rho in COS-7 cells. (A) p115-RhoGEF activity dominates the Tiam1-induced Rho inactivation. COS-7 cell lysates expressing Tiam1, p115GEF or both GEFs were assayed for active GTP-bound Rho and Rac by GTPase pulldown with GST-Rhotekin-GBD and GST-PAK-GBD, respectively. Rho and Rac proteins were detected by Western blotting using 26C4 and 23A8 antibodies. Aliquots of the lysates were probed with the same antibodies to control for total amounts of Rho. (B) PDZ-RhoGEF-mediated Rho activation is downregulated by Tiam1 activity. Rho and Rac pulldown assays of the cell lysates expressing PDZ-RhoGEF, Tiam1 or both GEFs assayed as described for (A). (C) PDZ-RhoGEF, p115-RhoGEF and Tiam1 were immunoblotted with antibodies against myc and HA tags to check their expression.

Results and discussion

Tiam1 interferes with PDZ-RhoGEF-mediated Rho activation

Several studies have demonstrated that Rac1 can negatively regulate the activity of RhoA in various cell lines (Kozma et al., 1997; Horwitz and Parsons, 1999; Sander et al., 1999; van Leeuwen et al., 1999; Zondag et al., 2000; Noren et al., 2001; Nimnual et al., 2003). To investigate the biochemical pathway of the Rac-induced inactivation of Rho, we utilized the COS-7 cell line because of its quantitative transfection efficiency (see materials and methods section). Expression of a constitutive active Tiam1 mutant encompassing the C-terminal 1199 amino acids (Michiels et al., 1995) in COS-7 cells resulted in a drastic increase in active GTP-bound Rac (Figure 1A). However, this did not cause any reduction in the Rho·GTP level, as shown by Rho and Rac GTPase pulldown assays. To overcome this problem, we set out to increase the basal level of Rho·GTP by transfecting COS-7 cells with constructs encoding full-length p115-RhoGEF and N-terminal truncated PDZ-RhoGEF lacking the PZD-RGS domains. Expression of both GEFs led to stimulation of the GDP/GTP exchange of Rho in COS-7 cells without changing the basal level of Rac (Figure 1A,B). Similar data have previously been shown for the expression of p115-RhoGEF in HEK293 cells (Wells et al., 2001). Upon coexpression of Tiam1, complete downregulation of Rho was observed in the presence of PDZ-RhoGEF, but interestingly not in the presence of p115-RhoGEF (Figure 1). To examine whether Tiam1 does have any effects on the expression of both RhoGEFs, it was coexpressed with p115-RhoGEF and PDZ-RhoGEF in COS-7 cells. Figure 1C shows that expression of the RhoGEFs was not affected by Tiam1, as there was no difference in their protein levels in the presence or absence of Tiam1.

These data demonstrate that Tiam1/Rac signaling induces Rho inactivation in COS-7 cells and provide the first evidence of different signaling properties between the two RhoGEFs that are differentially modulated by

Tiam1. However, it remains unclear whether Tiam1 directly interferes with the upstream signals that are required for activation of PDZ-RhoGEF, but not for p115-RhoGEF activation, or induces signals towards down regulation of active Rho·GTP, which cannot be accomplished when Rho is activated by the p115-RhoGEF function. In the former scenario, the RGS-like domain of p115-RhoGEF may play a role that is missing in the PDZ-RhoGEF used in this study. It is important to note that this PDZ-RhoGEF protein lacking the PDZ-RGS domains is able to activate serum response element (Fukuhara et al., 1999) and to induce actin stress fibers in human bladder carcinoma (Ruemenapp et al., 1999), which results from its RhoGEF activity.

Tiam1/Rac signaling downregulates active Rho·GTP through p190-RhoGAP

It has been proposed that the formation of a complex between p190-RhoGAP and p120-RasGAP is required for the recruitment of p190 to membrane fractions, where it downregulates Rho signaling (Chang et al., 1995; Hu and Settleman, 1997). To investigate if Rac activity is involved in a biochemical pathway that regulates the activation of a Rho-specific GAP such as p190, we coexpressed the N-terminal SH2 domain of p120-RasGAP (called SH2n) together with Tiam1 and PDZ-RhoGEF. As shown in Figure 2A, the Tiam1-induced inactivation of Rho is inhibited in the presence of the SH2n domain. This interferes neither with the GEF activity of PDZ-RhoGEF (Figure 2A) nor with Tiam1 (Figure 2B). Similar results were obtained with the second further C-terminal SH2 domain of p120 (data not shown), indicating that both SH2 domains might compete with endogenous p120-RasGAP for binding to the tyrosine-phosphorylated p190-RhoGAP.

To investigate whether there is a direct interaction between the SH2 domains of p120 and the tyrosine-phosphorylated p190, we overexpressed SH2n in the presence of either tyrosine kinase v-Src or Tiam1, respectively, in COS-7 cells. As shown in Figure 3, p190 coimmunoprecipitated with the SH2 domain of p120 most strongly in the presence of v-Src. These results are in part in good agreement with a recent report showing that the constitutive active mutant (G12V: glycine 12 to valine) of Rac1 expressed in HeLa cells is able to downregulate Rho by increasing tyrosine phosphorylation of p190 (Nimnual et al., 2003). In the absence of v-Src but in the presence of Tiam1, however, there is a low level of phosphorylated p190 detectable. However, this cannot be attributed to the Tiam1 activity (Figure 3, upper panel, lane 3) when compared to the SH2n-expressing cells (Figure 3, upper panel, lane 2). Nevertheless, these results indicate that the SH2n domain directly associates with the tyrosine-phosphorylated p190-RhoGAP and blocks complex formation between p190 and p120. The SH2 domains thus represent a dominant negative form of p120-RasGAP that interferes with the Tiam1/Rac-induced downregulation of Rho by p190-RhoGAP.

We further examined the role of p190-RhoGAP as a regulator of the Rac-induced Rho inactivation by modulating the phosphorylation state of this GAP using the activities of both the low-molecular-weight protein tyrosine phosphatase (LMW-PTP) and the tyrosine kinase inhibitor PP1. LMW-PTP has been shown to dephosphorylate p190-RhoGAP upon stimulation with platelet-derived growth factor (Chiarugi et al., 2000). The latter is available in the culture medium supplemented with 10% FCS (see materials and methods). Hence, we cotransfected COS-7 cells with a combination of LMW-PTP, Tiam1 and PDZ-GEF and performed a Rho pulldown assay. Figure 4A shows that the level of active Rho remained unchanged in the presence of PDZ-GEF, Tiam1 and LMW-PTP. These data were further supported by treatment of the transfected COS-7 cells with the Src-family-selective inhibitor PP1 (Figure 4B), which are consistent with phosphorylation of p190-RhoGAP being important for the Tiam1/Rac-induced downregulation of Rho in COS-7 cells.

As can be seen in Figure 4A, the basal level of the active GTP-bound Rho is further reduced in cells expressing LMW-PTP. One obvious reason is that LMW-PTP does not selectively modulate the phosphorylation state of p190-RhoGAP (Raugei et al., 2002). Apart from p190-RhoGAP, LMW-PTP shows substrate specificity for

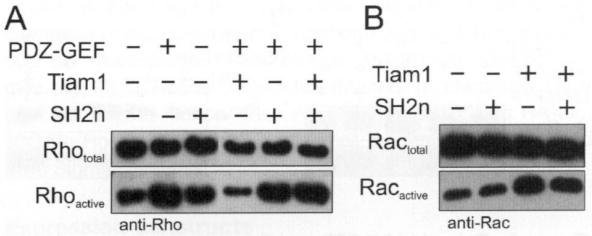

Figure 2 Dominant negative effect of the SH2 domain of p120-RasGAP on the Tiam1-induced inactivation of Rho.
Rho (A) and Rac (B) pulldown assays were performed as described in the legend of Figure 1A using COS-7 cell lysates expressing Tiam1, the N-terminal SH2 domain of p120-RasGAP (SH2n), or both Tiam1 and SH2n. The levels of Tiam1, PDZ-RhoGEF and SH2n are the same as shown in Figures 1C and 3.

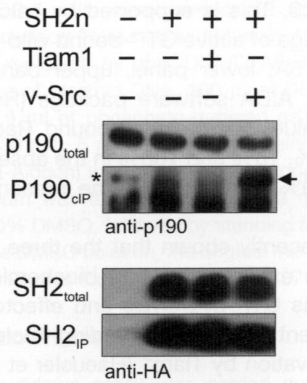

Figure 3 SH2 domain bound to p190-RhoGAP.
HA-SH2n was immunoprecipitated from COS-7 cell lysates expressing Tiam1, v-Src, Sh2n or a combination of Tiam1/SH2n or v-Src/SH2n. The samples were immunoblotted with antibodies against immunoprecipitated (IP) HA-SH2n and coimmunoprecipitated (coIP) p190-RhoGAP. Aliquots of the lysates were probed with the same antibodies to control for total amounts of Sh2n and p190. The arrow indicates the position of the phosphorylated p190-RhoGAP. The asterisk indicates a faint unspecific band at the position of phosphorylated p190.

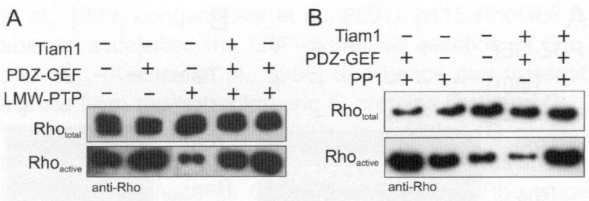

Figure 4 Role of LMW-PTP and Src-kinase.
(A) A Rho pulldown assay was performed as described in the legend of Figure 1A using COS-7 cell lysates expressing Tiam1, PDZ-RhoGEF, LMW-PTP and a combination of these constructs as indicated. (B) COS-7 cells transfected with Tiam1 and PDZ-RhoGEF were treated with 0.5 μM of the tyrosine kinase inhibitor PP1 for 30 min. Lysates were assayed for the active GTP-bound Rho as described in the legend of Figure 1A.

several different membrane tyrosine-kinase receptors, including platelet-derived growth factor (PDGF) receptor (Chiarugi et al., 1995), and for the focal adhesion kinase p125FAK (Giannoni et al., 2003). Dephosphorylation of these molecules may be the reason for the reduced basal level of active Rho in COS-7 cells, since both PDGF receptor and p125FAK have been shown to be upstream of Rho signaling (Hotchin and Hall, 1995; Defilippi et al., 1997; Sander et al., 1999).

Active Rho is downregulated by Rac1, but not by Rac2 or Rac3

Tiam1 is a known activator of Rac1 in cells (Bollag et al., 2000); however, its specificity for Rac2 and Rac3 was not known. To examine the nucleotide exchange activity of Tiam1 on Rac1, the respective constructs of wild-type Rac1, Rac2 and Rac3 were cotransfected with Tiam1 in COS-7 cells and cell lysates were analyzed in a Rac pull-down assay. Figure 5A shows that despite differences in the total amounts of the exogenous Rac proteins (upper band in the upper panel), Tiam1 does activate both Rac1 and Rac2 when comparing GTP-bound proteins in the presence and absence of Tiam1, but probably has no effect on Rac3. This is supported by calculation of the total percentage of active GTP-bound wild-type Rac proteins (Figure 5A, lower panel, upper bands) using the densitometric AIDA software package (Raytest, Berlin, Germany). Values for the GTP-bound Rac1, Rac2 and Rac3 were 3%, 16% and 103% in the absence of Tiam1, and 71%, 89% and 100% in the presence of Tiam1, respectively.

We have recently shown that the three highly related Rac isoforms exhibit consistent biochemical characteristics, such as GTP-hydrolysis and effector binding, as well as different properties regarding nucleotide binding and their activation by Tiam1 (Haeusler et al., 2003; Fiegen et al., 2004). Although Rac proteins share 92% sequence identity, the isolated GEF domain of Tiam1 was up to 30-fold more effective on Rac2 compared to Rac1 or Rac3. This is based on the different dynamics of these GTPases rather than deviations in their primary sequence (Haeusler et al., 2003). However, a higher specificity of Tiam1 for Rac2 versus Rac1 remains to be analyzed in monocytes or neutrophils, since Rac2 is expressed in hematopoietic cells rather than in COS-7 cells.

Rac3 is apparently constitutively active in the absence of exogenous Tiam1 and likely not regulated by Tiam1

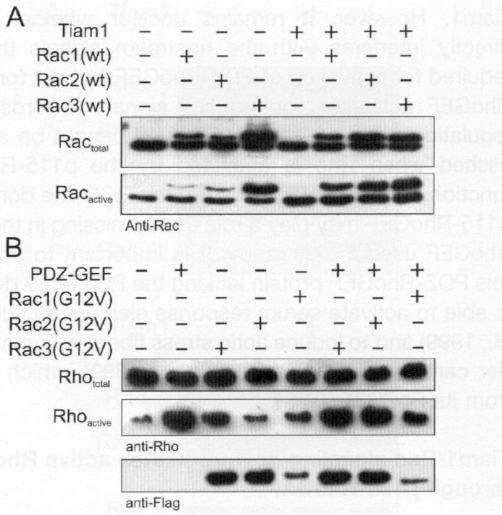

Figure 5 Tiam1/Rac1 signaling to Rho.
(A) Activation of the Rac isoforms by Tiam1 was analyzed in a Rac pulldown assay using COS-7 cell lysates expressing Tiam1 in combination with the wild-type (wt) Rac1, Rac2 and Rac3. Aliquots of the lysates were probed with an antibody against Rac to control for total amounts of the exogenous (upper band) and endogenous Rac proteins. (B) The effect of Rac isoforms on Rho inactivation was analyzed in a Rho pulldown assay as described in the legend of Figure 1A using COS-7 cell lysates expressing PDZ-RhoGEF in combination with the constitutive active mutants (G12V) of the Rac isoforms. Aliquots of the lysates were probed with antibodies against Rho and flag-tagged Rac isoforms to control for total amounts of these proteins.

(Figure 5A, lower panel, lanes 4 and 8). It has been shown to be hyperactive in breast cancer cells, too, where it controls proliferation via a PAK-dependent pathway (Mira et al., 2000). The reason for the high level of active Rac3 is unknown. However, it is important to note that the high level of GTP-bound Rac3 probably cannot only be attributed to a higher expression level of Rac3 (Figure 5A, upper panel, lane 4). Like most other small GTPases, Rac3 functionally acts as a molecular switch cycling between the inactive GDP-bound state and the active GTP-bound state (Haeusler et al., 2003). Both intrinsic reactions, nucleotide exchange and GTP-hydrolysis, are very slow and need to be regulated by GEFs and GAPs, respectively (Haeusler et al., 2003). We have previously shown that the nucleotide exchange reaction of Rac3 can be accelerated *in vitro* by the catalytic domain of Tiam1 (Haeusler et al., 2003), indicating that conversion of Rac3 into its active GTP-bound form requires activation of the proper GEF by the upstream signals. GAP function, on the other hand, resembles an enzyme, and catalytic amounts of the GAP are sufficient to quantitatively stimulate the GTP-hydrolysis reaction within seconds, presuming the proper GAP is localized to the same subcellular compartment as Rac3. To examine the sensitivity of Rac3 towards GAP, we measured the GTP-hydrolysis reaction of Rac3 in real time in the presence and absence of p50-RhoGAP using a novel kinetic method as recently described for Rac1 (Eberth et al., 2005). Accordingly, the GAP-stimulated GTPase reaction of Rac3 was comparable to that of Rac1 (data not shown), suggesting that the basic mechanism of the GAP-stimulated GTPase reaction of Rac3 is conserved.

Thus, future investigations will prove which process, activation by GEFs or inactivation by GAPs, is the reason for the elevated level of active GTP-bound Rac3. In another scenario, GDI binding to Rac3 may influence the level of active Rac3 in COS-7 cells, as it is also known to bind to the GTP-bound forms of the GTPase and to inhibit its intrinsic GTP-hydrolysis reaction (Nomanbhoy and Cerione, 1996).

To further address the question of which Rac isoforms might be responsible for the Tiam1-induced inactivation of Rho, we cotransfected COS-7 cells with PDZ-RhoGEF and the constitutive active mutants of Rac1, Rac2 and Rac3, respectively. As shown in Figure 5B, only Rac1(G12V) seems to be active in downregulating Rho, but not the other Rac isoforms, particularly when considering the total amounts of Rac1 protein compared to Rac2 and Rac3 (Figure 5B, lower panel). The latter may be explained by the recent finding that constitutively active Rac1 is degraded in cells by a proteasome-dependent pathway, while Rac2 and Rac3 are not subjected to degradation (Pop et al., 2004). Taking these results together, we conclude that Tiam1/Rac1 signaling in COS-7 cells regulates the p190-RhoGAP-mediated inactivation of Rho.

Conclusion

In the present study we addressed the role of p190-RhoGAP as the executive regulatory element that downregulates Rho in response to Tiam1/Rac1 signaling. We showed that interfering with phosphorylation of p190-RhoGAP or its association with the SH2 domain of p120-RasGAP can cut off its RhoGAP function in COS-7 cells. In addition, Rac1, but not Rac2 or Rac3, was the substrate of Tiam1 in a pathway that is able to switch off Rho activation by PDZ-RhoGEF, but not by p115-RhoGEF. The difference between these two RhoGEFs was observed for the first time in this study, which might result from N-terminal truncation of PDZ-RhoGEF, and awaits further investigation. Another important issue that remains to be addressed is the downstream effector that links Rac1 to p190-RhoGAP. One obvious candidate might be PAK1, as it antagonizes Rho/ROCK-induced actomyosin-generated tension and contractility of the migrating cell in response to active GTP-bound Rac. This scenario can be excluded, as it has been shown that an activated PAK1 mutant was unable to mediate Rho inactivation in NIH3T3 fibroblasts (Sander et al., 1999). Another effector candidate is NAD(P)H oxidase, which regulates the production of reactive oxygen species that represent an essential component in the Rac-mediated Rho inactivation and subsequent lamellipodia formation in HeLa cells (Nimnual et al., 2003).

Materials and methods

Reagents and antibodies

Reagents and antibodies were purchased from the following providers: tyrosine kinase inhibitor PP1, Alexis Biochemicals (Grünberg, Germany); monoclonal mouse antibody against Rac, 23A8, Upstate Biotechnology (Hamburg, Germany); monoclonal mouse antibody against RhoA, 26C4, Santa Cruz (Heidelberg, Germany); monoclonal mouse antibody against p190-RhoGAP, 30, BD Transduction Laboratories (Heidelberg, Germany); myc-tag, 9E10, Roche Diagnostics (Mannheim, Germany); Flag-tag, M2, Sigma-Aldrich (Deisenhofen, Germany); and HA-tag, 12CA5, Roche Diagnostics. All other reagents were obtained from Sigma-Aldrich.

Expression constructs

Human Rac1, Rac2 and Rac3 and their respective constitutive active G12V mutants were cloned in pcDNA3-Flag via BamHI/EcoRI sites. The SH2 domains of the human p120-RasGAP [amino acids (aa) 77–275 and aa 350–447] were cloned in pMT2sm-HA via SalI/XbaI. pGEX-PAK (aa 57–141), pGEX-Rhotekin (aa 1–90), pMT2-v-Src, pUTSV-HA-Tiam1 (C-terminal aa 393–1591 or C1199), pcDNA3-flag-PDZ-RhoGEF (aa 637–1522), pCAN-myc-p115-RhoGEF (full length) and pRcCMV-wtLMW-PTP were kindly provided by J. Collard (Michiels et al., 1995; Reid et al., 1999), K.H. Jakobs (Ruemenapp et al., 1999), G. Bollag (Hart et al., 1996) and P. Chiarugi (Chiarugi et al., 2000), respectively.

Preparation of the GST-fusion proteins

The GTPase-binding domains (GBDs) of PAK1 and Rhotekin were produced as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* for 4 h at 30°C with 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were washed and suspended in 50 mM Tris/HCl, pH 7.5. After sonication, the lysates were clarified by centrifugation and 1-ml aliquots of the supernatants were snap-frozen and stored at -80°C. Aliquots of 150 μl of the respective supernatants were coupled to 30 μl of glutathione-Sepharose beads at 4°C. After 30 min the beads were washed three times and used in a GTPase pulldown assay.

Cell culture and transient transfection assays

Monkey kidney epithelial COS-7 cells were cultured at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) supplemented with penicillin/streptomycin (Invitrogen), and 10% fetal calf serum (FCS; Invitrogen). Then 1.5×10^6 COS-7 cells were seeded on 10-cm^2 culture dishes 24 h prior to transfection. Transient transfections of COS-7 cells were carried out according to the DEAE-dextran method (Sussman and Milman, 1984). Briefly, the medium was replaced by the DNA/DEAE-dextran solution containing 5 μg of plasmid DNA, 100 μl of DEAE-dextran (10 mg/ml), and 1.9 ml of phosphate-buffered saline (PBS). After incubation for 30 min, 5 ml of medium containing 80 μM chloroquine (Sigma-Aldrich) was added for additional incubation for 3 h. The medium was then replaced with a medium supplemented with 10% DMSO, followed by standing for 2.5 min. After aspiration of the DMSO solution, the original medium was added to the transfected cells for 48-h incubation at 37°C and 5% CO_2.

GTPase pulldown assay

The GTPase pulldown assay was carried out as previously described (Reid et al., 1999; Sander et al., 1999; Fiegen et al., 2004). Briefly, 48 h after transfection, cells were washed in ice-cold PBS and lysed in ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 2 mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin). Two 10-cm^2 culture dishes were used for the Rac assay and three for Rho assays. Lysates (1.5 ml) were clarified by centrifugation and the supernatant was incubated for 20 min at 4°C with GST-fusions of PAK-GBD and Rhotekin-GBD coupled to glutathione-Sepharose beads. After washing (three times) with lysis buffer, bound proteins were denatured with 4×

SDS-Laemmli sample buffer (100 mM Tris/HCl, pH 6.8, 33% glycerol, 300 mM dithiothreitol, 6.7% SDS and 0.01% bromophenol blue) at 95°C and analyzed for active GTP-bound forms of Rac and Rho by Western blotting. The experiments were performed two or three times to verify the results.

Immunoprecipitation

Immunoprecipitations were carried out as described by Moran et al. (1991) in lysis buffer containing 0.2 mM sodium ortho-vanadate and 1 mM EDTA, pH 8.0. γ-Bind Sepharose (Amersham Pharmacia, Braunschweig, Germany) was coupled to the respective monoclonal antibody at 4°C. After 30 min the beads were washed three times. Transiently transfected COS-7 cells were washed in ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer. Lysates were clarified by centrifugation and the supernatants were incubated with γ-Bind Sepharose-coupled monoclonal antibody for 1 h at 4°C. After washing (three times) with 50 mM Tris/HCl pH 7.4, bound proteins were denatured with 4× SDS Laemmli buffer at 95°C and analyzed by Western blotting.

Acknowledgments

We gratefully thank A. Wittinghofer for discussions and continuous support, P. Stege for excellent technical assistance, and A. Berken, A. Eberth and J.P. ten Klooster for critical reading of the manuscript. U.H. was supported by the Max-Planck Society. The results are part of the Ph.D. thesis of U.H. at Heinrich-Heine University, Düsseldorf, Germany.

References

Bishop, A.L. and Hall, A. (2000). Rho GTPases and their effector proteins. Biochem. J. *348*, 241–255.

Bollag, G., Crompton, A.M., Peverly Mitchell, D., Habets, G.G., and Symons, M. (2000). Activation of Rac1 by human Tiam1. Methods Enzymol. *325*, 51–61.

Bouton, A.H., Kanner, S.B., Vines, R.R., Wang, H.C., Gibbs, J.B., and Parsons, J.T. (1991). Transformation by pp60src or stimulation of cells with epidermal growth factor induces the stable association of tyrosine phosphorylated cellular proteins with GTPase activating protein. Mol. Cell. Biol. *11*, 945–953.

Bryant, S.S., Briggs, S., Smithgall, T.E., Martin, G.A., McCormick, F., Chang, J.H., Parsons, S.J., and Jove, R. (1995). Two SH2 domains of p120 Ras GTPase activating protein bind synergistically to tyrosine phosphorylated p190 Rho GTPase activating protein. J. Biol. Chem. *270*, 17947–17952.

Chang, J.H., Gill, S., Settleman, J., and Parsons, S.J. (1995). c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190 RhoGAP, and p120 RasGAP following epidermal growth factor stimulation. J. Cell Biol. *130*, 355–368.

Chiarugi, P., Cirri, P., Taddei, L., Giannoni, E., Camici, G., Manao, G., Raugei, G., and Ramponi, G. (2000). The low M_r protein tyrosine phosphatase is involved in Rho mediated cytoskeleton rearrangement after integrin and platelet derived growth factor stimulation. J. Biol. Chem. *275*, 4640–4646.

Defilippi, P., Venturino, M., Gulino, D., Duperray, A., Boquet, P., Fiorentini, C., Volpe, G., Palmieri, M., Silengo, L., and Tarone, G. (1997). Dissection of pathways implicated in integrin-mediated actin cytoskeleton assembly. Involvement of protein kinase C, Rho GTPase, and tyrosine phosphorylation. J. Biol. Chem. *272*, 21726–21734.

Dovas, A. and Couchman, J.R. (2005). RhoGDI: multiple functions in the regulation of Rho family GTPase activities. Biochem. J. *390*, 1–9.

Driessens, M.H., Olivo, C., Nagata, K., Inagaki, M., and Collard, J.G. (2002). B plexins activate Rho through PDZ-RhoGEF. FEBS Lett. *529*,168–172.

Eberth, A., Dvorsky, R., Becker, C.F., Beste, A., Goody, R.S., and Ahmadian, M.R. (2005). Monitoring the real-time kinetics of the hydrolysis reaction of guanine nucleotide-binding proteins. Biol. Chem. *386*, 1105–1114.

Ellis, C., Moran, M., McCormick, F., and Pawson, T. (1990). Phosphorylation of GAP and GAP associated proteins by transforming and mitogenic tyrosine kinases. Nature *343*, 377–381.

Engers, R., Springer, E., Michiels, F., Collard, J.G., and Gabbert, H.E. (2001). Rac affects invasion of human renal cell carcinomas by upregulating tissue inhibitor of metalloproteinases TIMP 1 and TIMP 2 expression. J. Biol. Chem. *276*, 41889–41897.

Etienne Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629–635.

Fiegen, D., Haeusler, L.C., Blumenstein, L., Herbrand, U., Dvorsky, R., Vetter, I.R., and Ahmadian, M.R. (2004). Alternative splicing of Rac1 creates a self-activating GTPase. J. Biol. Chem. *279*, 4743–4749.

Fukuhara, S., Murga, C., Zohar, M., Igishi, T., and Gutkind, J.S. (1999). A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J. Biol. Chem. *274*, 5868–5879.

Fukuhara, S., Chikumi, H., and Gutkind, J.S. (2001). RGS containing RhoGEFs: the missing link between transforming G proteins and Rho? Oncogene *20*, 1661–1668.

Giannoni, E., Chiarugi, P., Cozzi, G., Magnelli, L., Taddei, M.L., Fiaschi, T., Buricchi, F., Raugei, G. and Ramponi, G. (2003). Lymphocyte function-associated antigen-1-mediated T cell adhesion is impaired by low molecular weight phosphotyrosine phosphatase-dependent inhibition of FAK activity. J. Biol. Chem. *278*, 36763–36776.

Habets, G.G., Scholtes, E.H., Zuydgeest, D., van der Kammen, R.A., Stam, J.C., Berns, A. and Collard, J.G. (1994). Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell *77*, 537–549.

Haeusler, L.C., Blumenstein, L., Stege, P., Dvorsky, R., and Ahmadian, M.R. (2003). Comparative functional analysis of the Rac GTPases. FEBS Lett. *555*, 556–560.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509–514.

Hart, M.J., Sharma, S., el Masry, N., Qiu, R.G., McCabe, P., Polakis, P., and Bollag, G. (1996). Identification of a novel guanine nucleotide exchange factor for the Rho GTPase. J. Biol. Chem. *271*, 25452–25458.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C., and Bollag, G. (1998). p115 RhoGEF, a GTPase activating protein for Gα12 and Gα13. Science *280*, 2112–2114.

Horwitz, A.R. and Parsons, J.T. (1999). Cell migration – movin' on. Science *286*, 1102–1103.

Hotchin, N.A. and Hall, A. (1995). The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular Rho/Rac GTPases. J. Cell Biol. *131*, 1857–1865.

Hu, K.Q. and Settleman, J. (1997). Tandem SH2 binding sites mediate the RasGAP RhoGAP interaction: a conformational mechanism for SH3 domain regulation. EMBO J. *16*, 473–483.

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G. and Sternweis, P.C. (1998). RhoGEF, a GTPase activating protein for Gα12 and Gα13. Science *280*, 2109–2111.

Kozma, R., Sarner, S., Ahmed, S. and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol. Cell. Biol. *17*, 1201–1211.

Longenecker, K.L., Lewis, M.E., Chikumi, H., Gutkind, J.S., and Derewenda, Z.S. (2001). Structure of the RGS like domain from PDZ RhoGEF: linking heterotrimeric G protein coupled signaling to Rho GTPases. Structure *9*, 559–569.

Mertens, A.E., Roovers, R.C., and Collard, J.G. (2003). Regulation of Tiam1/Rac signalling. FEBS Lett. *546*, 11–16.

Mira, J.P., Benard, V., Groffen, J., Sanders, L.C., and Knaus, U.G. (2000). Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. Proc. Natl. Acad. Sci. USA *97*, 185–189.

McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, G., Rozakis Adcock, M., Chen, L.B., and Pawson, T. (1993). The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J. *12*, 3073–3081.

Michiels, F. and Collard, J.G. (1999). Rho-like GTPases: their role in cell adhesion and invasion. Biochem. Soc. Symp. *65*, 125–146.

Michiels, F., Habets, G.G., Stam, J.C., van der Kammen, R.A., and Collard, J.G. (1995). A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature *375*, 338–340.

Moran, M.F., Polakis, P., McCormick, F., Pawson, T., and Ellis, C. (1991). Protein tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of p21ras GTPase activating protein. Mol. Cell. Biol. *11*, 1804–1812.

Nimnual, A.S., Taylor, L.J., and Bar Sagi, D. (2003). Redox dependent downregulation of Rho by Rac. Nat. Cell Biol. *5*, 236–241.

Nomanbhoy, T.K. and Cerione, R. (1996). Characterization of the interaction between RhoGDI and Cdc42Hs using fluorescence spectroscopy. J. Biol. Chem. *271*, 10004–10009.

Noren, N.K., Niessen, C.M., Gumbiner, B.M., and Burridge, K. (2001). Cadherin engagement regulates Rho family GTPases. J. Biol. Chem. *276*, 33305–33308.

Noren, N.K., Arthur, W.T. and Burridge, K. (2003). Cadherin engagement inhibits RhoA via p190RhoGAP. J. Biol. Chem. *278*, 13615–13618.

Perrot, V., Vazquez-Prado, J., and Gutkind, J.S. (2002). Plexin B regulates Rho through the guanine nucleotide exchange factors leukemia-associated Rho GEF (LARG) and PDZ-RhoGEF. J. Biol. Chem. *277*, 43115–43120.

Pop, M., Aktories, K., and Schmidt, G. (2004). Isotype specific degradation of Rac activated by the cytotoxic necrotizing factor 1. J. Biol. Chem. *279*, 35840–35848.

Raugei, G., Ramponi, G., and Chiarugi, P. (2002). Low molecular weight protein tyrosine phosphatases: small, but smart. Cell. Mol. Life Sci. *59*, 941–949.

Reid, T., Bathoorn, A., Ahmadian, M.R., and Collard, J.G. (1999). Identification and characterization of hPEM 2, a guanine nucleotide exchange factor specific for Cdc42. J. Biol. Chem. *274*, 33587–33593.

Ridley, A.J. (2001). Rho GTPases and cell migration. J. Cell Sci. *114*, 2713–2722.

Ridley, A.J., Self, A.J., Kasmi, F., Paterson, H.F., Hall, A., Marshall, C.J., and Ellis, C. (1993). Rho family GTPase activating proteins p190, Bcr and RhoGAP show distinct specificities *in vitro* and *in vivo*. EMBO J. *12*, 5151–5160.

Roof, R.W., Haskell, M.D., Dukes, B.D., Sherman, N., Kinter, M., and Parsons, S.J. (1998). Phosphotyrosine (p-Tyr)-dependent and -independent mechanisms of p190 RhoGAP p120 RasGAP interaction: Tyr-1105 of p190, a substrate for c Src, is the sole p-Tyr mediator of complex formation. Mol. Cell. Biol. *18*, 7052–7063.

Rossman, K.L., Der, C.J., and Sondek, J. (2005). GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. Nat. Rev. Mol. Cell Biol. *6*, 167–180.

Ruemenapp, U., Blomquist, A., Schworer, G., Schablowski, H., Psoma, A., and Jakobs, K.H. (1999). Rho specific binding and guanine nucleotide exchange catalysis by KIAA0380, a dbl family member. FEBS Lett. *459*, 313–318.

Sander, E.E., ten Klooster, J.P., van Delft, S., van der Kammen, R.A., and Collard, J.G. (1999). Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. J. Cell Biol. *147*, 1009–1022.

Scheffzek, K. and Ahmadian, M.R. (2005). GTPase activating proteins: structural and functional insights 18 years after discovery. Cell. Mol. Life Sci. *62*, 3014–3038.

Settleman, J., Narasimhan, V., Foster, L.C., and Weinberg, R.A. (1992). Molecular cloning of cDNAs encoding the GAP associated protein p190: implications for a signalling pathway from ras to the nucleus. Cell *69*, 539–549.

Sussman, D.J. and Milman, G. (1984). Short-term, high-efficiency expression of transfected DNA. Mol. Cell. Biol. *4*, 1641–1643.

Swiercz, J.M., Kuner, R., Behrens, J., and Offermanns, S. (2002). Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. Neuron *35*, 51–63.

Taya, S., Inagaki, N., Sengiku, H., Makino, H., Iwamatsu, A., Urakawa, I., Nagao, K., Kataoka, S., and Kaibuchi, K. (2001). Direct interaction of insulin-like growth factor-1 receptor with leukemia-associated RhoGEF. J. Cell Biol. *155*, 809–820.

Uhlenbrock, K., Eberth, A., Herbrand, U., Daryab, N., Stege, P., Meier, F., Friedl, P., Collard, J., and Ahmadian, M.R. (2004). The RacGEF Tiam1 inhibits migration and invasion of metastatic melanoma via a novel adhesive mechanism. J. Cell Sci. *117*, 4863–4871.

van Aelst, L. and D'Souza Schorey, C. (1997). Rho GTPases and signalling networks. Genes Dev. *11*, 2295–2322.

van Leeuwen, F.N., van Delft, S., Kain, H.E., van der Kammen, R.A., and Collard, J.G. (1999). Rac regulates phosphorylation of the myosin II heavy chain, actinomyosin disassembly and cell spreading. Nat. Cell Biol. *1*, 242–248.

Vetter, I.R. and Wittinghofer, A. (2001). The guanine nucleotide binding switch in three dimensions. Science *294*, 1299–1304.

Wells, C.D., Gutowski, S., Bollag, G., and Sternweis, P.C. (2001). Identification of potential mechanisms for regulation of p115 RhoGEF through analysis of endogenous and mutant forms of the exchange factor. J. Biol. Chem. *276*, 28897–28905.

Wells, C.D., Liu, M.Y., Jackson, M., Gutowski, S., Sternweis, P.M., Rothstein, J.D., Kozasa, T., and Sternweis, P.C. (2002). Mechanisms for reversible regulation between G13 and Rho exchange factors. J. Biol. Chem. *277*, 1174–1181.

Zondag, G.C., Evers, E.E., ten Klooster, J.P., Janssen, L., van der Kammen, R.A., and Collard, J.G. (2000). Oncogenic Ras downregulates Rac activity, which leads to increased Rho activity and epithelial mesenchymal transition. J. Cell Biol. *149*, 775–782.

Received July 26, 2005; accepted December 23, 2005