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The invisible hand: regulation of RHO GTPases by RHOGDIs

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Preface

The 'invisible hand' is a term originally coined by Adam Smith in the *Theory of Moral Sentiments* to describe the forces of self-interest, competition, and supply and demand that regulate the resources in society. This metaphor continues to be used by economists to describe the self-regulating nature of a market economy. The same metaphor can be used to describe the RHO-specific guanine nucleotide dissociation inhibitor (RHOGDI) family, which operates in the background, as an invisible hand, using similar forces to regulate the RHO GTPase cycle.

Introduction

RHO family GTPases control a wide variety of cellular processes, including cell adhesion, migration and proliferation (Box 1). At any given time, only a small fraction of all RHO GTPases present in the cell are in the active state and are associated with membranes. The inactive pool is maintained in the cytosol by associating with RHO-specific guanine nucleotide dissociation inhibitors (RHOGDIs).

Despite the wide diversity in the RHO GTPase family, there are only three genes encoding RHOGDI in mammals (Box 2) ¹. RHOGDI1 (also known as RHOGDI α) is the most abundant and best-characterized member of the family; it is ubiquitously expressed and interacts with several RHO GTPases, including RHOA, RHOC, RAC1, RAC2 and CDC42 ^{2,3}. RHOGDI2 (also known as RHOGDI β , Ly-GDI and D4-GDI) is expressed in high levels by haematopoietic cells^{4,5}, but has been also found to be expressed in other tissues, as well as by cancer cells (see below). RHOGDI2 associates with several RHO GTPases *in vitro*, but with significantly lower affinity than RHOGDI1⁶. However, many of these interactions have not been detected *in vivo*⁷. RHOGDI3 (also known as RHOGDI γ) is the most divergent of the three and contains a unique amino-terminal extension that targets it to the Golgi complex and other cellular membranes⁸. RHOGDI3 is usually expressed at low levels and seems to interact predominantly with RHOB and RHOG⁸⁻¹⁰. Completing the catalogue of binding specificities of RHOGDIs to the different RHO GTPases, which is far from comprehensive, should shed some light on the differences in the functions of the different isoforms.

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Although originally considered to be passive regulators, recent studies have shown that the RHOGDI family have a key role in the regulation of RHO GTPases. Like an 'invisible hand'—a term originally coined by Adam Smith in the *Theory of Moral Sentiments* to describe the forces of self-interest, competition and supply and demand that regulate the resources in society¹¹—RHOGDIs operate in the background, controlling key aspects of the RHO GTPase cycle. In this Review, we discuss how RHOGDIs regulate RHO GTPases, by modulating the extent of their expression, their membrane localization and their activation state. We also describe the mechanisms by which the interaction between RHOGDIs and RHO GTPases is regulated, and how, in many cases, this regulation can be selective for each type of RHO GTPase. Changes in the levels of expression of the different RHOGDIs have marked effects on the overall levels and activities of RHO GTPases and have been correlated with several types of cancer.

RHOGDI functions

RHOGDIs were initially characterized as simply RHO GTPase inhibitors; however, recent work indicates that their function is more complex¹².

RHOGDIs as negative regulators of RHO GTPases

The first RHOGDI (RHOGDI1) was originally discovered in the pre-genomics era, when new proteins were still identified on the basis of their biochemical properties and function. RHOGDI (or RHOB p20 GDI, as it was named then) was initially purified from rabbit intestine and later cloned from bovine brain tissue^{2, 13}. It was characterized as a protein capable of inhibiting some of the basic features of RHO proteins, (and not of RAS, RAP and RAB), such as the release of GDP and the loading of GTP to RHO-GDP. RHOGDIs do not prevent loading of GDP or GTP to a nucleotide-free RHO; this means that they inhibit the release of the nucleotide and not its binding to the RHO GTPase¹³. It was therefore clear that its biochemical activity was different from that of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). RHOGDIs were also found to inhibit the GTPase activity of RHO proteins, preventing both intrinsic and GAP-stimulated hydrolysis of GTP¹⁴. This dual inhibitory function would later be understood from the structure of RHOGDI complexed with a RHO GTPase. The N-terminal domain of RHOGDI interacts with the switch I and switch II domains of the RHO protein (see below) and restricts the spatial flexibility that is required for the transition between different nucleotide-bound forms of the RHO GTPase, essentially 'locking up' the GTPase. Conceptually, RHOGDI seemed to function in the RHO switch (Box1) simply as an inhibitory molecule that bound mostly to inactive RHO GTPases and preventing nucleotide exchange. This view of RHOGDI as an inhibitory regulator was extended by the observation that RHOGDI can extract RHO proteins from membranes and hold them in a complex in the cytosol away from their sites of action at membranes³.

RHOGDIs as chaperones

Given that RHO GTPases are prenylated and act at membranes, it is somewhat surprising that the largest fraction of each family member is found in the cytosol^{12, 15}. One of the main functions of RHOGDIs is to maintain a stable soluble pool of inactive RHO GTPases, which at any given time may account for most (90–95%) of the RHO proteins in the cell^{12, 15}. A generally accepted explanation for this cytosolic pool is that it acts as a reservoir, allowing inactive RHO GTPases to be rapidly translocated to any membrane in the cell for activation in response to specific signals. This 'instant deployment' strategy would allow cells to respond quickly to any stimulus or challenge that requires the activation of a particular RHO GTPase. It is important to note here that most of the studies have examined the effects on

the cytosolic pool of the major RHO GTPases — RAC1, RHOA and CDC42 — and findings may not apply to all family members.

One of the problems associated with maintaining a soluble RHO GTPase pool is the hydrophobic isoprenoid moiety at the carboxyl terminus of all RHO proteins. Isoprenylation of RHO proteins is essential for their proper subcellular localization and signalling¹⁶. However, in the absence of membranes, the isoprenoid moiety, which is highly hydrophobic, impairs the ability of RHO GTPases to fold properly¹². Binding to RHOGDI stabilizes the cytosolic RHO pool and shields the isoprenyl group from exposure to water by inserting it into a hydrophobic pocket in the C-terminal half of the molecule. Consistent with this, phosphorylation of RHOA on Ser188 increases its affinity for RHOGDI and protects it from degradation (see below)¹⁷.

This function of RHOGDIs has always been considered to be more of a 'housekeeping' role and has not been characterized in detail. However, it was recently found that, in the absence of RHOGDI1, the cytosolic pool of RHO GTPases is unstable and rapidly degraded in a proteasome-dependent manner (Fig. 1)¹². In the absence of the isoprenyl group, RHO GTPases are much more stable, although their signalling is impaired because they fail to localize to their sites of action at specific membranes. In this way, RHOGDIs act as chaperones, not as much in assisting the folding of newly synthesized proteins (although it is possible that this occurs), but by stabilizing the mature RHO GTPases when they are not associated with membranes. Even though a chaperone function for RHOGDIs has been long recognized¹⁸, their role in protecting against degradation was generally overlooked.

RHOGDI shuttle RHO GTPases between membranes

Since the discovery that RHOGDIs can extract RHO proteins from membranes, the idea that RHOGDIs may shuttle RHO GTPases between different membrane compartments has been prevalent. However, evidence has been presented both for and against this, and the debate continues. In a series of papers, Wilson-Delfosse and colleagues¹⁹⁻²¹ designed RHO GTPase mutants (Arg66Glu in RAC1 and CDC42, Arg68Glu in RHOA) that cannot bind to RHOGDIs and hypothesized that, if RHOGDI is required to shuttle RHO proteins between membranes, the mutated proteins should be mislocalized and not functional. By expressing constitutively active RHO GTPases, they found no significant difference in the signalling between the forms that were able or unable to bind to RHOGDIs. Similarly, RHOGDI-null cells responded the same way as their wild-type counterparts when constitutively active mutants of RAC1 and CDC42 were overexpressed^{19, 20}. This led them to conclude that RHOGDIs are not required for the translocation of RHO GTPases to their membrane destinations¹⁹⁻²¹. Consistent with this, yeast cells lacking Rdi1 (the RHOGDI orthologue) and RHOGDI1-knockout mice had mild phenotypes²²⁻²⁴. The *RD11* deletion yeast strain was indistinguishable from wild-type cells in terms of growth, cell morphology and mating, but exhibited defects in pseudohyphal growth and mitosis exit²². Similarly, RHOGDI1-knockout mice reached adulthood normally, suggesting there are other mechanisms that can control the cycling of RHO GTPases^{23, 24}.

By contrast, Cerione's group found that, although a fast-cycling mutant of CDC42 (Phe28Leu) induced cell transformation, this property was lost when a second mutation (Arg66Ala) that impaired binding to RHOGDI was introduced²⁵. CDC42 Phe28Leu can undergo spontaneous GTP-GDP exchange while maintaining full GTPase activity²⁶, and, unlike GTPase-defective CDC42 mutants (which are toxic to cells), can be stably expressed and exhibit properties of oncogenic transformation. Unable to associate with RHOGDI, the CDC42 double mutant, accumulated in membranes in the perinuclear region rather than reaching the plasma membrane²⁵. Similarly, another group found that the Arg66Glu RAC1 mutant, which cannot bind RHOGDI, failed to translocate to the plasma membrane in

response to hepatocyte growth factor (HGF) stimulation (which normally activates RAC1)²⁷. Fractionation of cells has revealed a biphasic distribution of membrane-associated RHOA, RAC1 and CDC42, with most found in the endoplasmic reticulum (ER) and a smaller amount of each one in the plasma membrane fraction. Depletion of RHOGDI1, however, causes a marked reduction in the levels of each GTPases that are associated with the plasma membrane¹², suggesting that RHOGDIs are involved in the transport of RHO GTPases to the plasma membrane.

How can this contrasting evidence be reconciled? One possibility is that there are other RHOGDI-like molecules compensating for the loss of RHOGDIs. For example, recent work shows that caveolin 1 binds to CDC42 and sequesters it in its GDP-bound form at secretory granules²⁸. CDC42 is required for insulin granule exocytosis, during which it is proposed to function in targeting the secretory granules to specific sites at the plasma membrane²⁹. Upon stimulation by glucose, CDC42 is dissociated from caveolin 1 and subsequently activated, allowing the secretory granules to fuse with the plasma membrane. Mechanistically, caveolin 1 assumes some of the properties of RHOGDIs by sequestering inactive CDC42 and restricting fusion of secretory granules. It will be interesting to discover whether other membrane-bound RHOGDI-like molecules contribute to the spatial and temporal regulation of RHO GTPases.

However, there is evidence against this theory: in the absence of RhoGDIs, essentially no RHO GTPases are detected in the cytosol¹². An alternative explanation, which we favour, comes from studies in yeast, in which there is evidence supporting the coexistence of two mechanistically distinct recycling systems for CDC42, a fast-cycling mechanism involving Rdi1 and a slower one involving vesicle trafficking (Figure 1)³⁰. At the early stages of bud formation, CDC42 is targeted to a small region in the plasma membrane that later becomes the bud site, where it functions to orchestrate the polarization of the actin cytoskeleton (which is required to direct secretory traffic to the budding site)³¹. Deletion of the Rdi1 significantly decreases the rate of exchange of CDC42 between the plasma membrane and the cytosol³⁰ but has no obvious growth phenotype, owing to the presence of the endocytic recycling pathway. However, when both pathways are inhibited, the polarization of CDC42 at the bud site is rapidly lost.

We favour the idea of vesicle trafficking accounting for the small amount of RHO proteins that can reach the plasma membrane in mammalian cells lacking RHOGDI1. The existence of two pathways by which RHO GTPases reach their membrane destinations provides an explanation for how constitutively active RHO proteins that cannot interact with RHOGDI can still exert their effects^{19, 20}. Without binding to RHOGDI, a fraction of RHO GTPases continues to be transported to the plasma membrane through vesicle trafficking; this occurs at a reduced rate, but one sufficient to account for their phenotype. We suspect that the mild phenotype of the RHOGDI1-knockout mice also results from sufficient transport of RHO proteins occurring through vesicle trafficking.

Structural analysis of RHOGDI–RHO GTPase

RHOGDI1 has been crystallized in complex with CDC42, RHOA and RAC1³²⁻³⁴ (Figure 2), whereas the structure of RHOGDI2 has been solved in complex with RAC2³⁵. These structures provide snapshots of the key residues involved in regulating the interactions and have provided the information necessary for mutagenesis studies that characterized the importance of these residues.

The structure of RHOGDIs comprises two main domains: a C-terminal domain (amino acids 74–204) — which includes the geranylgeranyl-binding pocket and is required to extract

RHO GTPases from the membrane — and a 'regulatory arm' at the N-terminus (amino acids 5–55) — which inhibits exchange and hydrolysis through interactions with the switch I and switch II domains in the RHO GTPases. The isoprenyl-binding domain adopts an immunoglobulin-like fold, and the surface of the geranylgeranyl-binding pocket is lined with hydrophobic residues^{32,34}. Insertion of the isoprenyl moiety perturbs the structure of the RHOGDI, displacing the β -strands β H and β I by 2.0Å, as well as several individual amino acids, to overcome steric hindrances imposed by the isoprenyl moiety. The N-terminal region of RHOGDIs, which is flexible and disordered when in solution, folds into two antiparallel helices upon formation of the complex and interacts with the switch I and switch II domains of the RHO GTPases (Figure 2)^{32,34,35}.

It is interesting to note that RHOGDIs can accommodate both GTP- and GDP-bound forms of RHO GTPases^{18,36}. Structural studies have shown that the main interaction sites between RHOGDIs and RHO GTPases are virtually unaffected by the nucleotide state³⁷.

Numerous key residues have been identified in the interface between RHOGDIs and RHO GTPases, including Thr35, Tyr64, Arg66, His103 and His104 (amino acid numbering refers to RAC1 and CDC42), which form hydrogen bonds with RHOGDIs. Thr35, located in the switch I domain, is particularly important for the interaction, as it is conserved in all GTPases and is required for the coordination of Mg^{2+} (which is essential for stabilizing the nucleotide). The regulatory arm in RHOGDIs interacts with Thr35 in CDC42 by forming a hydrogen bond with Asp45. Arg66, which is located in the switch II region and forms hydrogen bonds with Asp185, Pro30 and Ala31, is another key residue in the RHOGDI - RHO GTPase interaction; substitution of the RHO GTPase Arg66 for Glu (or of Arg68 in RHOA) abolishes the interaction with RHOGDIs¹⁹⁻²¹. Interestingly, the RHOGDI- RHO GTPase interaction can be rescued with a charge-reversing substitution in Asp185 in RHOGDI (Asp185Arg)²⁰.

Based on structural and experimental data, a two-step mechanism has been proposed to explain how RHOGDIs extract RHO GTPases from the membrane³⁶. First, the N terminus of the RHOGDIs associates with the switch I and switch II domains of the RHOGTPase. This initial binding step is followed by a slower isomerization event that results in the isoprenyl group swapping from the membrane to the hydrophobic pocket^{32,38}. The acidic patch in the RHOGDI geranylgeranyl-binding pocket may contribute to the extraction by competing with acidic phospholipids for binding to the polybasic tail of the RHO GTPase. It was recently shown that, in the presence of membranes, RHOGDIs have a much higher affinity for CDC42-GDP (inactive) than for CDC42-GTP (active)³⁸. By selectively extracting the GDP-bound (inactive) forms of RHO GTPases from membranes, RHOGDI could contribute to the concentration of active GTPases at the membrane.

Regulation of RHOGDI-RHO GTPase interactions

Selective activation of a single RHO GTPase by a signalling pathway requires the release of that RHO family protein from the RHOGDI. But here there is a conundrum because each of the three mammalian RHOGDIs interacts with multiple RHO GTPases. How can a single RHO GTPase be released from the RHOGDI selectively? In addition, our recent findings argue that displacement of a RHO GTPase from RHOGDI has to be coupled to its association with cell membranes (or some other entity) to prevent its degradation¹². Based on similar G proteins, such as members of the RAB family, the existence of a GDI dissociation factor (GDF) has been postulated. By definition, a GDF interacts with RHOGDIs and promotes the dissociation of the RHOGDI-RHO GTPase complex, rendering the RHO GTPase available for activation by GEFs. However, in contrast to the RAB system, a conserved GDF for RHO proteins has not been found. Evidence for a range of mechanisms

has been provided, which are discussed below. Some of them regulate the specific displacement of a single RHO GTPase from RHOGDI, whereas others displace all the RHO GTPases equally.

Release by lipids

Early work revealed that RHOGDI–RHO GTPase complexes could be disrupted by acidic lipids³⁹, and subsequent work showed that phosphoinositide lipids could open up RHOGDIs, thereby facilitating GEF-mediated exchange on bound RHOA⁴⁰. A role for lipids in the release of RHO GTPases from RHOGDI was shown during integrin-mediated adhesion^{41,42}. In suspended cells RAC1-GTP was found to be retained in the cytosol complexed with RHOGDI; however, integrin-mediated adhesion could mediate the dissociation of RAC1 from the RHOGDI. A lipid raft seemed to be responsible for the recruitment of RAC1 to sites of integrin engagement, and liposomes that were similar in lipid composition to lipid rafts were found to dissociate Rac1-GTP from RHOGDI⁴¹.

Another study using liposomes found that prenylated RAC1 bound to RHOGDI was inaccessible for nucleotide exchange mediated by the DBL homology domain (DH domain) and pleckstrin homology domain (PH domain) of the GEF T lymphoma invasion and metastasis-inducing 1 (TIAM1). However, nucleotide exchange could be observed when liposomes were added, and this corresponded specifically to a fraction of RAC1 dissociating from RHOGDI and binding to the liposomes⁴³. On the basis of these results, the authors proposed a two-step model for the activation of RHO GTPases: their release from RHOGDIs is promoted by lipids (FIG. 3a), which then leads to their activation by GEFs.

To examine this model further, another group measured exchange using liposomes of defined composition with RHOGDI bound to prenylated RAC1 and active DH and PH domains of the GEFs TIAM1 or TRIO. They found that liposomes containing phosphoinositides co-operated with active GEFs to promote the release of RAC1 from the RHOGDI⁴⁴. Like the previous group, these investigators favoured a two-step model, although the nature of their experimental conditions could not rule out a one-step model in which the GEF activity promoted dissociation of the RHOGDI–RAC1 complex.

Interestingly, in these experiments it was observed that if GTP was replaced with GDP, RAC1 did not dissociate from the RHOGDI and did not become associated with the liposomes⁴⁴. This is consistent with the finding that, in the presence of membranes, RHOGDI has a lower affinity for GTP-bound RHO GTPases than GDP-bound ones³⁸. Nevertheless, exchange did occur in the RHOGDI complex, indicating somewhat unexpectedly that the GEFs used in this experiment (TIAM and TRIO) could act on RAC1 complexed with RHOGDI. This observation suggests a one-step mechanism of RHO GTPase activation, but one in which the bound nucleotide (GTP versus GDP) is crucial for RHO GTPase release.

Release by GEFs

RHOGEFs have always been considered natural candidates for the role of GDFs in the RHO GTPase system. They act directly downstream of the dissociation event, and because they discriminate between individual RHO proteins and are targets of the signalling pathways that activate RHO GTPases, they could provide specificity to the reaction. However, according to structural analysis, both RHOGEFs and RHOGDIs interact with the switch I and switch II domains of RHO GTPases, which suggests that the two interactions may be mutually exclusive^{32, 45}.

Although findings indicate that GEFs can function as GDFs⁴⁴, in particular in the RAB GTPase subfamily^{46, 47}, there is little evidence suggesting that GEFs catalyze displacement

directly. Furthermore, even though a role for GEFs has been proposed in the two-step mechanism of RHO GTPase activation (see above), the two steps are mechanistically distinct⁴³; more work is needed to establish whether RHOGEFs have direct roles as GDFs or whether their actions are confined to the second step, activating the RHO protein that has been released from RHOGDI into the membrane.

Release by specific protein interactions

Another possibility is that specificity in the dissociation of the RHOGDI–RHO GTPase complex is afforded by distinct protein–protein interactions. For example, ezrin, radixin, and moesin (ERM) proteins, p75 neurotrophin receptor (p75^{NTR}; also known as NGFR) and the Tyr kinase ETK have all been reported to promote the displacement of RHO GTPases from RHOGDI⁴⁸⁻⁵¹ (FIG.3b). The N terminus of ERM proteins associates with RHOGDIs and can compete for binding to all RHO GTPases tested, thereby inhibiting RHOGDI function and promoting the activation of RHO proteins^{50, 52}. However, *in vivo* data suggest that ERM proteins may regulate the preferential release of RHOA^{50, 53}. Interestingly, radixin also interacts with the GEF DBL, suggesting it may couple release of the GTPase from RHOGDI with activation⁵².

p75^{NTR} is a neurotrophin receptor that functions in the regulation of axonal elongation by neurotrophins and by several myelin-derived proteins. Neurotrophins stimulate neurite outgrowth by inhibiting RHOA activity, whereas myelin-derived proteins activate RHOA and inhibit axonal elongation. The ability of p75^{NTR} to modulate RHOA activity depends on its ability to interact with RHOGDI to promote RHOA release and subsequent activation⁵¹. Myelin-derived proteins, such as neurite outgrowth inhibitor (NOGO) or myelin-associated glycoprotein (MAG), enhance the association of p75^{NTR} with RHOGDIs, which suggests that they activate RHOA by inducing its release from the RHOGDI (Figure 3)⁵¹.

ETK is a non-receptor Tyr kinase that belongs to the Bruton Tyr kinase (BTK) family and has been shown to have a role in the regulation of various cellular processes, including cytoskeletal reorganization and cell motility⁵⁴. ETK promotes the specific displacement of RHOA (but not RAC1 and CDC42) from RHOGDI1 in a kinase-independent manner⁴⁸. ETK interacts with RHOA through an N-terminal PH domain and competes for its binding to RHOGDIs, promoting its release and subsequent activation⁴⁸.

Release by phosphorylation

In recent years, phosphorylation has emerged as one of the key post-translational modifications that can selectively alter the affinities of the different RHO GTPases for RHOGDIs for one another. In general terms, phosphorylation of RHOGDIs decreases their affinity for RHO GTPases, promoting RHO GTPase release and making them available for activation. By contrast, phosphorylation of RHO GTPases increases their affinity for RHOGDIs, promoting their sequestration and inactivation in the cytosol (Table 1).

This suggests that, at least to a certain extent, some kinases can function as bona fide GDFs. For example, phosphorylation of RHOGDI1 on Ser101 and Ser174 by p-21-activated kinase (PAK1) reduces its affinity for RAC1 but not RHOA, promoting the release of RAC1 and its subsequent activation⁵⁵. Similarly, phosphorylation of RHOGDI1 on Ser34 by protein kinase C α (PKC α) selectively releases RHOA (but not RAC1 or CDC42) and promotes its activation⁵⁶. Ser101 and Ser174 are located in the hydrophobic binding cleft of RHOGDI on its solvent-exposed surface, suggesting their phosphorylation could perturb binding of the isoprenyl moiety of the RHO GTPase³² (FIG. 2). However, as the phosphorylation-mediated release is specific for RAC1, it is possible that interactions with the polybasic region, the most divergent sequence between RHO GTPases, may also be affected by the negative

charge afforded by the phosphorylation and contribute to release. Ser34 is located in the N-terminal regulatory arm of RHOGDIs, which interacts primarily with the switch II domain of RHO GTPases³². This region folds into a helix-loop-helix motif that is maintained mainly by hydrophobic interactions, so it is possible that the negative charge from the phosphate group interferes with the RHO GTPase interaction (FIG. 2). In addition, Ser34 is surrounded by many key residues in the N terminus of the RHOGDI, which interact with Arg66 (in CDC42 and RAC1), a residue in RHO GTPases that is essential for the interaction with RHOGDIs (and correlates with Arg68 in RHOA, RHOB and RHOC)¹⁹⁻²¹. It is therefore possible that phosphorylation of Ser34 disrupts or interferes with the interactions surrounding Arg66, triggering displacement. However, Arg66 is conserved in most RHO GTPases, so it is not clear how Ser34 affects specifically RHOA but not RAC1 and CDC42 release.

It is tempting to speculate that a RHOGDI phosphorylation code (similar to the histone modification code) controls which RHO GTPase is to be released from the complex in response to a stimulus. This attractive idea, which has been previously postulated¹, would solve the requirement for a GDF in an elegant way. However, this hypothesis is hard to justify based on the sequence and structural data. The residues that are phosphorylated in RHOGDIs are either concentrated around the geranylgeranyl-binding pocket or in the regulatory arm region that interacts primarily with the switch II region in the RHO GTPase (FIG. 2 and Table 1), so interfering with binding to the prenylated region of RHO GTPases should affect all RHO proteins the same way. The same can be argued for the switch II region, which is identical in the major RHO GTPases (RHOA, RAC1 and CDC42).

An alternative hypothesis is that selective release of a particular RHO GTPase from the RHOGDI is achieved by the recruitment of the complex to a specific cellular location, where the RHOGDI is phosphorylated. This could be mediated by scaffolding proteins that, in response to a signal, can recruit a particular RHOGDI– RHO GTPase complex to a site of stimulation. A specific kinase would then phosphorylate the RHOGDI, triggering the release of the RHO GTPase and allowing its subsequent activation by a RHOGEF. Consistent with this, a recent report showed that, upon platelet-derived growth factor treatment, diacylglycerol kinase- ζ (DGK ζ) forms a complex with RAC1 and RHOGDI and promotes the release of RAC1. DGK ζ stimulates the production of phosphatidic acid, which induces PAK activity. PAK subsequently phosphorylates RHOGDI, releasing RAC1 for activation⁵⁷ (FIG. 3c). In DGK ζ -deficient fibroblasts PAK1 phosphorylation and RHOGDI–RAC1 dissociation were attenuated and PAK failed to target properly to focal adhesions.

Similarly, following stimulation by HGF, DGK α was found to promote the formation of phosphatidic acid, which in this case recruits atypical PKC ζ (aPKC ζ)– aPKC ι , in complex with RHOGDI and RAC1. The activation of aPKC ζ – aPKC ι mediates the release of RAC1 from the RHOGDI, allowing its activation²⁷.

Another example involves the transmembrane receptor syndecan4, a heparan sulfate proteoglycan. Syndecan4 and its adaptor, synectin (which binds to the intracellular tail of syndecan 4), form a complex with RHOGDI⁵⁸, an interaction that increases RHOGDI's affinity for RHOG. Binding of syndecan4 to its ligand, fibroblast growth factor 2 (FGF2), promotes the activation of PKC α , which in turn phosphorylates RHOGDI. This mediates the release of RHOG (but not of RAC1), leading to its activation and further downstream effects^{59, 60}.

RHOGTPase crosstalk through RHOGDIs

Most RHO GTPases bind to at least one of the three RHOGDIs that are expressed in mammals¹. In addition, it has been previously shown that the total amount of the most

abundant RHOGDI is roughly equivalent to the sum of the levels of the three major RHOGTPases: RAC1, CDC42 and RHOA⁶¹. We have recently shown that RHO proteins can only exist in complex with RHOGDI or associated with cellular membranes¹². These results imply that the total levels of the major RHO proteins are determined by the amount of RHOGDIs in the cell. The limited amount of RHOGDIs generates competitive pressure between the different GTPases to bind to RHOGDI to avoid degradation (FIG. 1). At steady state, the RHO GTPases must reach an equilibrium between their ability to bind RHOGDI, the competition from the other RHO GTPases and their turnover by degradation. This previously unnoticed crosstalk between RHO GTPases through RHOGDI predicts that any change in the level of a particular RHO GTPase, or in the affinity of a particular RHO GTPase for RHOGDI, whether physiologically or experimentally induced, should interfere with this equilibrium.

We have recently shown that the exogenous overexpression of a single RHO GTPase competes with endogenous RHO proteins and displaces them from RHOGDI in a dose-dependent manner, triggering their subsequent degradation¹². In addition, the remaining membrane-bound RHO proteins are eventually extracted by RHOGDIs, displaced by the overexpressed protein and degraded. The result is the virtual silencing of both the protein levels and activity of all endogenous RHO proteins associated with RHOGDIs.

In spite of this result, there is evidence that in some situations the released RHO proteins may become activated. For example, a recent study observed that increased PKG-mediated phosphorylation of Ser188 in RHOA in smooth muscle cells increased the binding of RHOA to RHOGDI and displaced RAC1⁶². However, the released RAC1 became activated, leading to cell migration. A similar situation was observed in PtK1 epithelial cells, in which PKA activity was observed to be synchronized with protrusion at the leading edge⁶³. PKA-mediated phosphorylation of RHOA on Ser188 increased its affinity for RHOGDI and reduced its activity. It will be interesting to learn whether a similar displacement and activation of RAC1 is involved in this situation too.

This could have a significant role when the levels of a particular RHO GTPase or RHOGDI are affected, as occurs in many cancer types (see below), by indirectly affecting the homeostasis of the RHO GTPase system as a whole, magnifying the effects of altered expression levels.

Biological roles of RHOGDIs

The only known functions of RHOGDIs are in the context of their interactions with and regulation of RHO GTPases (for reviews on RHO GTPase functions, see Refs^{64, 65}). Briefly, some of the best-characterized effects of RHO GTPases are on the cytoskeleton. For example, RHOA, RAC1 and CDC42 can all stimulate actin polymerization by interacting with different effectors. The resulting phenotypes are typically, although not always, distinct: RAC1 promotes cell spreading, inducing the formation of lamellae at the cell periphery; CDC42 induces the formation of filopodia; and RHOA induces the formation of filamentous actin bundles, such as stress fibres, although RHOA-induced lamellae have also been described in some cell types⁶⁶. These three ubiquitously expressed RHO family members exert other effects on the cytoskeleton and coordinately regulate many types of cell migration. RHO GTPases also play important parts in cell adhesion (both to other cells and to the matrix), cell polarity, vesicle trafficking, cell cycle progression, gene expression and differentiation. Indeed, there seem few cellular activities in which RHO proteins do not have some regulatory role^{64, 65}.

When RHOGDI1 has been investigated by knockdown of protein expression or gene knockout, the overall levels of all RHO GTPases with which it interacts decrease. There was

a loss of detectable cytosolic RHO proteins, but the remaining membrane-bound fraction of each RHO protein is highly activated¹². A similar situation was seen in mammalian cells, when RHOGDI1 was knocked down, and in yeast when the single RHOGDI was deleted^{12, 22, 24}. Given these results, a prominent phenotype might be expected; however, surprisingly, there is only a mild phenotype in yeast and mice^{22, 24}. In mice, the loss of RHOGDI1 does not affect embryonic development, but the adult mice develop progressive renal defects, that ultimately lead to death and are attributed to increased RAC1 activity²³. In addition, the male mice are infertile, with impaired spermatogenesis, and there are problems with implantation of RHOGDI1-null embryos in female mice²⁴. One possible explanation for the mild phenotype is that RHOGDI2 compensated for the loss of RHOGDI1. However, the double knockout of RHOGDI1 and RHOGDI2 produces a phenotype that is only slightly more severe than the single RHOGDI1 knockout, with additional immunological defects superimposed on those induced by the loss of RHOGDI1⁶⁷.

Although mice lacking RHOGDIs are viable and develop normally, some effects have been observed when the behaviour of individual cells has been examined. For example, knockdown of RHOGDI1 in bladder cancer cells increased their migration, but knockdown of RHOGDI2 had no effect⁶⁸. By contrast, silencing RHOGDI1 in a rapidly migrating melanoma cell line inhibited cell migration¹². At first sight, these contradictory results seem difficult to reconcile. One potential explanation relates to the two modes of cycling RHO proteins to the plasma membrane (see above). In the absence of RHOGDIs, the slow delivery of RHO proteins to the plasma membrane by vesicle trafficking may be sufficient to satisfy the requirements of slowly migrating cells but inadequate for those migrating rapidly. The fact that slow cells actually increase their speed of migration is consistent with the remaining fraction of RHO proteins (particularly RAC1) being highly activated when RHOGDIs are depleted. So, although the amount reaching the plasma membrane is greatly decreased, those that do get to this site are activated and stay at the membrane for longer, thus stimulating migration. By contrast, fast-migrating cells may need to recycle RAC1 or other RHO proteins to newly formed protrusions at a speed that exceeds that of vesicle trafficking.

Other phenotypic changes have also been observed in cells depleted of RHOGDI1. For example, mesangial cells from the knockout mice revealed decreased rates of cell spreading⁶⁹. When vascular permeability was studied in RHOGDI1-knockout mice, an increase in the basal permeability of the pulmonary vascular endothelium was observed⁷⁰. This correlated with increased activity of RHOA in these cells and opening of the interendothelial junctions.

RHOGDIs and cancer

Changes in RHOGDI1 and RHOGDI2 expression levels have been associated with many cancers⁷¹. However, the changes vary depending on the tumour type. For instance, RHOGDI1 expression is upregulated in colorectal and ovarian cancer, and in this case high expression levels correlates with increased invasion and resistance to chemotherapy⁷²⁻⁷⁴. By contrast, RHOGDI1 expression is reduced in brain cancers and correlates with reduced expression of RHOA and RHOB but not RAC1. The expression levels of the three proteins are inversely correlated with the degree of malignancy⁷⁵. In breast cancers, conflicting results have been reported, with RHOGDI1 expression being increased or decreased in different studies^{76, 77}. Finally, RHOGDI1 is downregulated in hepatocellular carcinoma, and its expression was found to be controlled in part by a recently characterized microRNA, miR-151, which is localized in an intron of the gene encoding focal adhesion kinase (FAK) and is expressed with it⁷⁸. During hepatocellular tumour progression, FAK expression

increases together with miR-151, promoting the downregulation of RHOGDI1 and facilitating tumour invasion and metastasis⁷⁸.

The expression levels of RHOGDI2 are also severely altered in a range of cancers^{79, 80}. RHOGDI2 expression is increased in pancreatic cancers and this correlates with increased invasiveness^{80, 81}. The opposite occurs in bladder cancers, in which RHOGDI2 is downregulated in invasive tumour cells^{68, 82}, and this decrease correlates with decreased patient survival⁸³. Similarly, RHOGDI2 is lost in Hodgkin's lymphoma cell lines, although here there is no clear correlation with growth or survival⁸⁴. In breast tumours the pattern of expression of RHOGDI2 is complex. There is evidence that RHOGDI2 expression is biphasic, increasing at the early stages of progression but decreasing sharply during metastasis⁸⁵. RHOGDI has also been linked to oestrogen receptor expression and was identified as part of a predictive gene signature for poor prognosis for tumours that express oestrogen receptor⁸⁶.

The effects of the RHOGDIs on cancers are clearly complex and do not fit a simple explanatory model. It has to be remembered that the consequences of changes in their expression are manifested through their actions on multiple RHO GTPases, and that the levels and activities of these vary significantly in different cell types and different cancers. Even a single RHO family member can have opposite effects in different tumour types. This is well illustrated by work on the RAC-specific GEF TIAM1: although it was originally discovered in a search for genes that promoted T cell invasion and metastasis⁸⁷, in epithelial cells it has the opposite effect, delaying migration but enhancing stable cell-cell junctions⁸⁸. In both cases, the effects were due to the TIAM1-mediated activation of RAC1, which, actually, has different effects in the two cell types. As the interaction of RHOGDI with RHO GTPases is also governed by phosphorylation on multiple sites, the activities of specific kinases will likely affect how RHOGDI affects the phenotype of particular cancer cells.

Conclusion

In this Review we have discussed some of the emerging ideas about RHOGDIs, how they act as chaperones for RHO GTPases, regulating RHO protein stability, and how RHOGDIs shuttle the GTPases to membranes for activation and interaction with their downstream targets.

Understanding the factors that control the release of RHO proteins from RHOGDIs is one of the unresolved but exciting areas in the field. We anticipate that answers to the questions in this area will come from continued biochemical analysis of this process, using reconstituted *in vitro* model systems, as well as from imaging techniques that allow the visualization of RHOGDI complexes in living cells.

Another topic of considerable interest relates to the crosstalk between RHO GTPases that occurs through their competitive binding to RHOGDIs. The recent work showing that phosphorylation of RHOA by PKA increases its binding affinity to RHOGDI1 and results in displacement and activation of RAC1⁶², illustrates a level of interaction between RHO proteins that had not been anticipated. The discovery of other examples of crosstalk mediated by RHOGDIs will strengthen the idea that they contribute actively to RHO protein dynamics and signalling and are not simply passive inhibitors of the RHO GTPase cycle.

Finally, little is known about how the expression of RHOGDI1 or RHOGDI2 is regulated. The fact that the level of RHOGDI1 seems to be related to the sum of the expression levels of the major RHO proteins suggests that there are feedback mechanisms coordinating the expression of both the RHO GTPases and RHOGDIs. The discovery that miR-151 regulates RHOGDI1 expression opens a new window on this problem⁷⁸. We suspect that other factors

influencing the expression of the RHOGDIs remain to be discovered and that many of these may be altered in cancer cells. RHOGDIs have been suggested as potential targets for cancer therapy⁷¹, but if this direction is pursued it will be crucial to learn more about the factors regulating RHOGDI expression particularly in tumour cells.

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Glossary

Prenylated	Modified by the attachment of an isoprenoid to a C terminal cysteine residue.
Isoprenoid	Compounds that are derived from isoprene (2-methyl-1,3-butadiene) linked together in head-to-tail or tail-to-tail conformations and that comprise farnesyl and geranylgeranyl molecules that are used in the covalent modification of proteins on cysteine residues.
Isoprenylation	A post-translational modification of proteins by the attachment of an isoprenoid to a C-terminal Cys. The isoprenoids used, farnesyl diphosphate or geranylgeranyl diphosphate, are derived from the same biochemical pathway that produces cholesterol.
geranyl-geranyl	A 20-carbon isoprenoid precursor to geranyl-geranylated proteins. The source of the geranyl-geranyl group is geranyl-

	geranyl pyrophosphate, an intermediate in the HMG-CoA reductase pathway used by organisms in the biosynthesis of terpenes and terpenoids.
switch I and II	Two regions in RHOGTPases that undergo a conformational change in the active GTP-bound form and provide a platform for the selective interaction with downstream effectors.
immunoglobulin-like fold	A type of protein domain that consists of a 2-layer sandwich of between 7 and 9 antiparallel β -strands arranged in two β -sheets.
steric hindrance	occurs when the size of groups within a molecule prevents chemical reactions that are observed in related smaller molecules.
Isomerization	The process by which one molecule is transformed into another molecule that has exactly the same molecular formula, but with a different structure.
DH domain (Dbl homology)	A domain of about 200 amino acids that has been shown to encode GEF activity specific many RHO family members.
PH domains (Pleckstrin homology)	A protein domain of approximately 120 amino acids that occurs in a wide range of proteins involved in intracellular signaling. PH domains frequently bind to cell membranes by interacting with acidic phospholipids, such as phosphoinositides.
Biphasic	Having two distinct phases.
Lipid raft	Membrane microdomains that are enriched in cholesterol, sphingolipids and lipid-modified proteins such as GPI-linked proteins and palmitoylated proteins. These microdomains often function as platforms for signalling events.

Biographies

RGM Biography: Rafael Garcia-Mata obtained his PhD from the University of Alabama at Birmingham in 2002, and after postdoctoral research at the University of North Carolina at Chapel Hill joined the faculty at the University of North Carolina where he is now Assistant Professor and Simmons Scholar. The research in his lab is centered in understanding the mechanisms of regulation of RHO family of small GTPases.

RGM Lab webpage: <http://rgmlab.webnode.com/>

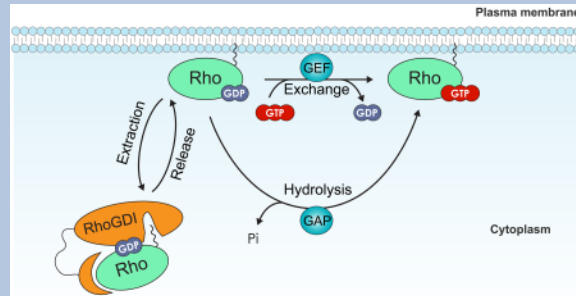
EB Biography: Etienne Boulter obtained his Ph.D. in Cell Biology from the University of Nice Sophia-Antipolis, Nice, France, and followed with a postdoctoral training at The University of North Carolina at Chapel Hill. He is currently is a Postdoctoral Fellow at the University of Nice Sophia-Antipolis, Nice, France, where he studies the regulation of Rho proteins by CD98hc in the epidermis.

KB's biography: Keith Burrige obtained his Ph.D. from Cambridge University and after postdoctoral research at Cold Spring Harbor Laboratory joined in 1981 the faculty at the University of North Carolina, Chapel Hill, where he is currently Kenan Distinguished Professor of Cell and Developmental Biology. He has a longstanding interest in cell adhesion and the cytoskeleton, and has worked for many years on various aspects of RHO GTPase signaling.

KB Lab webpage: <http://www.med.unc.edu/cellbio/faculty-research/burrige>

Box 1**The RHO switch**

RHO proteins are guanine nucleotide-binding proteins (G proteins also known as GNBPs) that belong to the larger group of GTPases, which include heterotrimeric G proteins, elongation factors, tubulins, septins and large GTPases such as dynamin. Biochemically, GTPases are hydrolase enzymes that bind and hydrolyze GTP. In a similar way to ATP, GTP can act as an energy carrier, but it also has an active role in signal transduction, particularly in the regulation of G protein activity. G proteins, including RHO GTPases, are molecular switches that cycle between an inactive GDP-bound and an active GTP-bound conformation (see the figure). The transition between the two conformational states occurs through two distinct mechanisms: activation by GTP loading and inactivation by GTP hydrolysis. GTP loading is a two-step process that requires the release of the GDP-bound nucleotide and its replacement by a GTP molecule. Nucleotide release is a spontaneous but slow process that has to be catalyzed by RHO guanine-nucleotide exchange factors (RHO GEFs), which associate with RHO GTPases and trigger the release of the nucleotide. The resulting nucleotide-free binary complex has no particular nucleotide specificity. However, the cellular concentration of GTP is markedly higher than that of GDP, which favours GTP loading, resulting in the activation of RHO GTPase. Conversely, to turn off the switch, GTP has to be hydrolyzed. This is facilitated by RHO GTPase-activating proteins (RHOGAPs), which stimulate the intrinsically slow hydrolytic activity of RHO proteins. It is worth noting that, although GEFs and GAPs are the canonical regulators of this cycle, several alternative mechanisms, such as post-translational modifications, may fine-tune the RHO switch. In addition, inactive RHO GTPases are extracted by RHO-specific guanine nucleotide dissociation inhibitors (RHOGDIs) from cell membranes to prevent their inappropriate activation and to protect them from misfolding and degradation.



Box 2**RHOGDI evolution and orthologues**

Guanine nucleotide dissociation inhibitors (GDIs) can be categorized according to their guanine nucleotide-binding protein (G protein) specificity, but not all G proteins have GDIs (for example Ras does not, but the RHO and Rab families do). The different GDI families share some common functional and structural features, but they usually have unrelated amino acid sequences, suggesting that the families emerged independently during evolution. The RHOGDI family (see the figure) is defined by a conserved RHOGDI backbone. In humans, there are three RHOGDI proteins: RHOGDI1 (also known as RHOGDI α and encoded by ARHGDI A), which is expressed ubiquitously; RHOGDI2 (also known as RHOGDI β , Ly-GDI, D4-GDI and encoded by ARHGDI B), which is expressed by haematopoietic cells among others and is commonly upregulated in certain tumours; and RHOGDI3 (also known as RHOGDI γ and encoded by ARHGDI G), which is expressed mostly in the brain. Because RHOGDIs are evolutionarily conserved proteins, they have several orthologues in other eukaryotes (see the figure). There is a single RHOGDI in many unicellular organisms such as *Saccharomyces cerevisiae* (Rdi1), and also in protostomes such as *Caenorhabditis elegans* (RHI-1) and *Drosophila melanogaster* (RHOGDI). Other metazoans have two or three RHOGDIs: for example, *Danio rerio* has two and *Xenopus laevis* has three. Tetrapods, including avians and mammals, generally have three RHOGDIs. Interestingly, plants such as *Arabidopsis thaliana* have their own particular set of Rho proteins (ROPs) and their own specific RHOGDI proteins, which highlights both their differences with the animal kingdom and the universal necessity to have RHOGDIs despite these differences. From the study of RHOGDIs in different organisms, it emerges that RHOGDIs have fundamentally conserved functions in eukaryotes: the regulation of RHO protein cycling between lipid membranes and the cytosol, the stabilization of RHO proteins and the inhibition of nucleotide release. The figure was generated by aligning the sequences of the RHOGDIs using ClustalW and by compiling node distances using the neighbour-joining method.



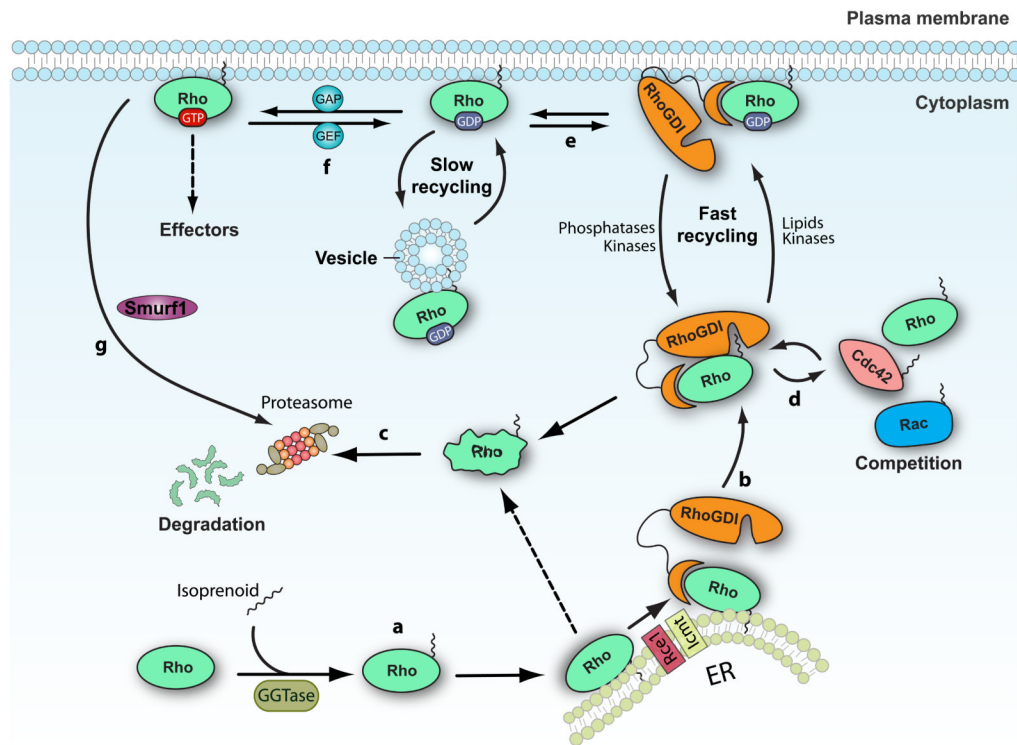


Figure 1. The RHO GTPase cycle

(a) Newly synthesized RHO family GTPases are geranylgeranylated and then post-translationally modified by the protease Ras-converting enzyme 1 (RCE1) and by isoprenylcysteine carboxyl methyltransferase (ICMT) at the cytoplasmic face of the endoplasmic reticulum (ER). (b) After geranylgeranylation, RHO proteins associate with RHO specific guanine nucleotide dissociation inhibitors (RHOGDIs), which sequester them in the cytosol and protect them from degradation. (c) Free prenylated cytosolic RHO GTPases are unstable and are rapidly degraded by the proteasome. (d) Several RHO GTPases can associate with RHOGDI and compete for its binding. Overexpression of a GTPase can displace the endogenous RHO proteins from RHOGDI targeting them for degradation. (e) The rate of cycling of the RHOGDI -RHO GTPase complex between the cytosol and the membrane can be regulated by post-translational modifications on both the RHO GTPases and the RHOGDI, which modulate the affinity of the interaction. A slower pathway for recycling RHO proteins through vesicle trafficking has also been postulated. (f) Once at the membrane, the RHO GTPases can be activated by guanine nucleotide exchange factors (GEFs) and bind to downstream effectors. Following inactivation by GTPase-activating proteins (GAPs), RHO GTPases are extracted from the membrane by RHOGDI. (g) Active RHOA can also be targeted for degradation by the ubiquitin ligase SMAD ubiquitylation regulatory factor 1 (Smurf1). GGTase, geranylgeranyl transferase.

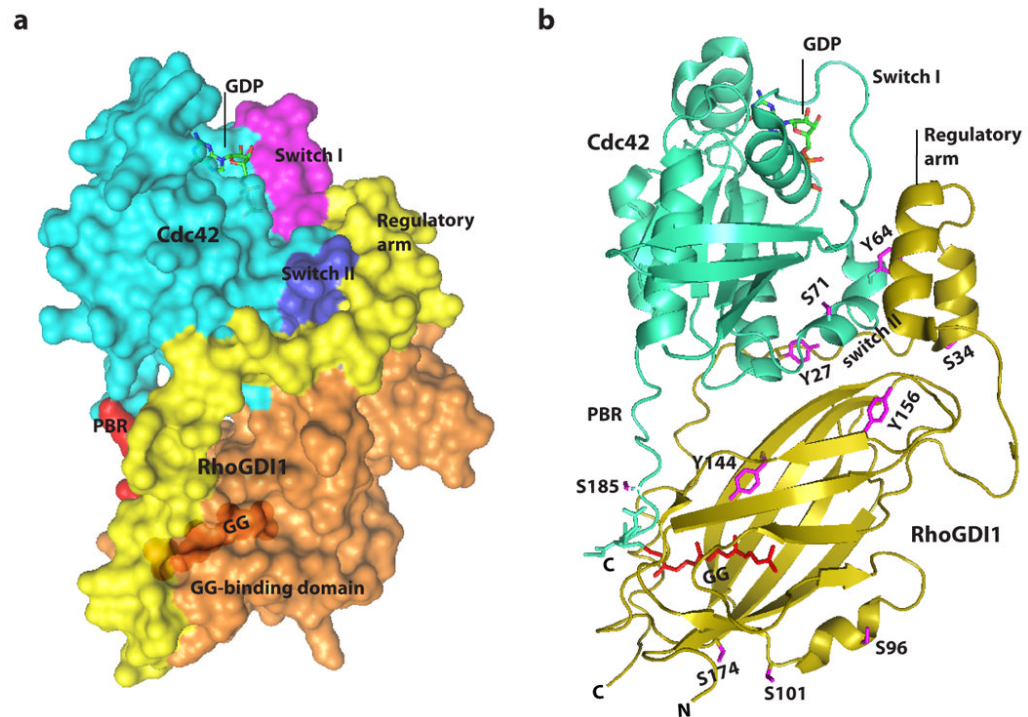


Figure 2. Structure of the RHO GDI-RHO GTPase complex

(A) Space filling model showing CDC42 in complex with RHO-specific guanine nucleotide dissociation inhibitor 1 (RHOGDI1)³². The domains that participate in the interaction are highlighted and labelled on the structure. (B) Cartoon representation of the crystal structure of prenylated CDC42 in complex with RHOGDI1³². The phosphorylated residues are shown in magenta, CDC42 is shown in green, and RHOGDI1 is shown in gold. These structures are reproduced from the [NCBI Molecular Modeling Database \(ID: 1275\)](#). C, carboxyl terminus; GG, geranylgeranyl; N, amino terminus; PBR, Poly Basic Region.

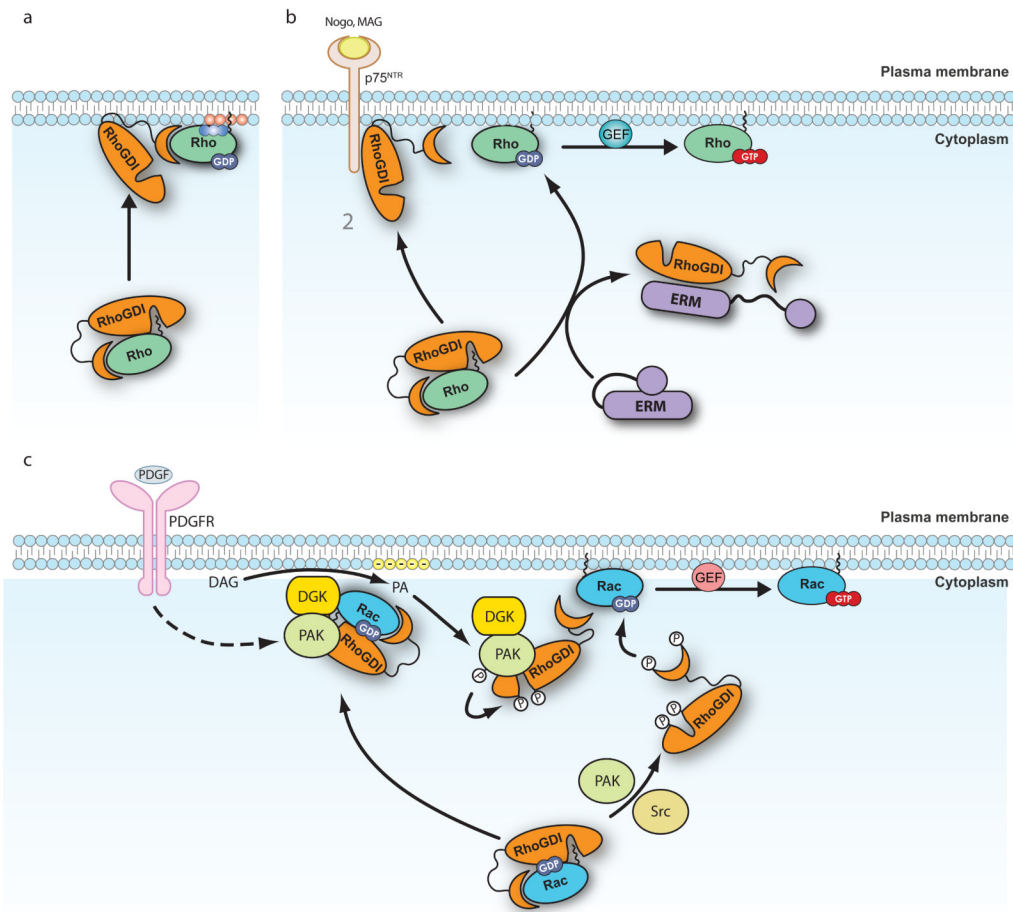


Figure 3. Mechanisms of regulation of the RHO GDI-RHO GTPase interaction

a | Release by lipids: The presence of acidic phospholipids can promote the release of RHO GTPases from RHO-specific guanine nucleotide dissociation inhibitors (RHOGDIs). Phospholipids mediate a partial opening of the complex that exposes the GTPases to RHO-specific guanine nucleotide exchange factors (RHOGEFs) or other dissociation factors such as the ones described in b. **b | Release by protein-protein interactions:** p75 neurotrophin receptor (p75^{NTR}) and ezrin, radixin and moesin (ERM) proteins interact with RHOGDIs and facilitate the release of RHOA, which can then be activated by specific RHOGEFs. The interaction of p75^{NTR} with RHOGDIs is enhanced by myelin derived proteins such as myelin-associated glycoprotein (MAG) and neurite outgrowth inhibitor (NOMO). ERM proteins also need to be activated to interact with RHOGDI. **c | Release by phosphorylation.** Phosphorylation of RHO GDI Ser, Thr and Tyr residues promote the release of RHO GTPases. Depending on the residues phosphorylated, this release can be specific for a single RHO GTPase, or affect multiple RHO GTPases simultaneously (Table 1). For example p21-activated kinase (PAK)- or FER-mediated phosphorylation promote the specific release of RAC1, but not RHOA or CDC42. Some kinases act in concert to target the RAC1-RHO GDI complex to the membrane and regulate the local release of RAC1 at specific site on the membrane, where it is subsequently activated by RHOGEFs. Diacylglycerol kinase- ζ (DGK ζ) forms a complex with PAK, RAC1 and RHOGDI. In response to platelet derived growth factor (PDGF), DGK ζ stimulates the production of phosphatidic acid (PA), which induces PAK activity. Active PAK then phosphorylates RHOGDI and releases RAC1 for

activation. Positively charged phospholipids are indicated in blue, negatively charged phospholipids in red. DAG, diacylglycerol; PDGFR, PDGF receptor.

Table 1
Post-translational modifications that modulate the RHOGDI-RHO GTPase interaction

Phosphorylation site	Kinase	GDI or GTPase	Effect	Refs
RhoGDIs [‡]				
Ser 34	PKC α	GDI1	Promotes dissociation of RhoA	56,89 [§]
Ser 96	PKC α	GDI1	Promotes dissociation of RhoA and RhoG	58,90
Ser 101	PAK1	GDI1	Promotes dissociation of Rac1 (and to a lesser extent Cdc42)	55
Ser 174	PAK1	GDI1	Promotes dissociation of Rac1 (and to a lesser extent Cdc42)	55
Ser 174	PKA	GDI1	Inhibits interaction with RhoA	91
Ser 148	ND	GDI1	ND	92
Thr ?	PKC ζ	GDI1	Promotes dissociation of RhoA, Rac1 and Cdc42	93
Tyr 27 ^{//}	Src	GDI1/2	Promotes dissociation of RhoA, Rac1 and Cdc42	94,95
Tyr 156 ^{//}	Src	GDI1/2	Promotes dissociation of RhoA, Rac1 and Cdc42	94,95
Tyr 156 [¶]	Fer	GDI1	Promotes dissociation of Rac1	96
RhoGTPases				
Ser188	PKA/A/PKG/SLK	RhoA	Increased association with GDI	97-102
Ser187	PKA	RhoG	Increased association with GDI	101
Ser185	PKA	Cdc42	Increased association with GDI	103
Tyr64	Src	Cdc42	Increased association with GDI	104
Ser71	Akt	Rac1, Cdc42	Increased association with GDI	105,106

ND, not determined; PAK1, p21-activated kinase 1; PK, protein kinase; RHOGDI, RHO-specific guanine nucleotide dissociation inhibitor; SLK, STE20-like kinase.

* Amino acid numbering corresponds to the sequence of human RHOGDI1.

[‡] Indicates RHOGDI1 residues; Tyr27, Ser34, Ser148 and Tyr156 are conserved in RHOGDI2 and RHOGDI3, whereas RHOGDI1 Ser101 and Ser174 are Thr in RHOGDI2, and Ser96 is present only in RHOGDI1.

[§] This article gave the first description of RHOGDI phosphorylation by PKC α . However, the phosphorylated residue was not identified.

^{//} The equivalent Tyr residues in RHOGDI2 (Tyr24, Tyr153) are also phosphorylated by SRC⁹⁵. Mainly Tyr156 is phosphorylated, with Tyr27 being only weakly phosphorylated.

[¶] The phosphorylated residue is in the carboxy terminal half of RHOGDI1, where Tyr156 is located.