Structural and biochemical investigation of the activity, specificity and regulation of regulators (GEFs & GAPs) of the Rho family

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Düsseldorf, im November 2012

To my family

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Publications

This thesis is based on the following publications:

Jaiswal, M., Dubey, B.N., Koessmeier, K.T., Gremer, L., and Ahmadian, M.R. (**2012**). *Biochemical assays to characterize Rho GTPases*. **Methods Mol Biol** *827*, 37-58.

Jaiswal, M., Fansa, E. K., Dvorsky, R., Ahmadian, M. R. (**2012**). *New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm*. **Biol Chem**. (submitted)

Jaiswal, M., Gremer, L., Dvorsky, R., Haeusler, L.C., Cirstea, I.C., Uhlenbrock, K., and Ahmadian, M.R. (**2011**). *Mechanistic insights into specificity, activity, and regulatory elements of the regulator of G-protein signaling (RGS)-containing Rho-specific guanine nucleotide exchange factors (GEFs) p115, PDZ-RhoGEF (PRG), and leukemia-associated RhoGEF (LARG)*. **J Biol Chem** *286*, 18202-18212.

Jaiswal, M., Dvorsky, R., Ahmadian, M.R., *Deciphering the molecular and functional basis of Dbl family proteins: a meta-analysis approach.* **(in press)**

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Jaiswal, M., Risse. S., Cirestea I.C., Olayioye, M.A., Ahmadian, M.R., *Identification of structural and functional determinants of the inhibition of DLC tumor suppressor protein by p120 RasGAP.* **(in preparation)**

1. Introduction

Biology is a broad field of science to study about life and living organism whereas the basic structural and functional unit of life is cell. All living organism from plant to animals are composed of cells, which perform several metabolic functions and required for storage, support, growth, transport of resources or defense of the organism. Each and every cellular function needs to be tightly regulated and any failure of regulatory events leads to imbalance and several disorder to cell and therefore to the organism as whole. In all multicellular organisms this communication between cells is carried on by signaling process and signals between the cells are transmitted via various groups of regulatory molecules present inside and outside the cell. There is considerable variation in the structure and function of the different types of regulatory molecules that serves as signal transmitters and proteins are one of them. Among proteins, small GTPases (guanosine triphosphatases) are one of the crucial signal transmitters and they participate as central control elements in signal transduction that touch each and every aspect of cell biology.

Small GTPase belongs to the superclass of Guanine nucleotide binding proteins (GNBPs) or G-proteins or GTPases, which defined by their ability to bind and to exchange the guanine nucleotides: GTP (guanosine triphosphate) or GDP (guanosine diphosphate), and to hydrolyze GTP. On the basis of their unique set of sequences and structural signatures all available GNBPs are divided in two major classes: i) TRAFAC and ii) SIMIBI. The TRAFAC class (translation factors) includes translation factor, hetrotrimeric G proteins (HTGPs) and monomeric small GTPase/Ras superfamily as the most prominent members whereas the SIMIBI class (signal recognition particle, MinD and BioD) includes the signal recognition particle (SRP), its receptor (SR) and a few other families (Leipe et al., 2002; Wittinghofer and Vetter, 2011). Ras superfamily is the founding member of small GTPases and consists of a single subunit (monomeric) with molecular mass of 20-25 kDa. Like larger heterotrimeric G-proteins, they cycle between active GTP-bound state and an inactive GDP-bound state, thereby acting as molecular switches in signal transduction pathways. The human Ras (rat sarcoma) superfamily contains more than 150 small GNBPs, which are subdivided into five subfamilies on the basis of sequence and functional similarities: Ras, Rho, Rab, Ran and Arf (Takai et al., 2001; Vigil et al., 2010) and control each particular aspect of cell metabolism (Bourne et al., 1990), such as cell proliferation for Ras (Hancock and Parton, 2005), cell morphology

for Rho (Wennerberg and Der, 2004), vesicle trafficking for Rab and Arf (Donaldson and Honda, 2005; Bucci and Chiariello, 2006), and nuclear trafficking for Ran (Pemberton and Paschal, 2005). The three Ras genes HRas, KRas and NRas were first discovered as oncogene in rat sarcoma virus and neuroblstoma (Downward, 1990). Originally identified as Ras-homologous proteins, members of the Rho family came to attention of researchers when it was discovered that they regulate cytoskeleton reorganization in 1991 (Ridley, 2001a). Main focus of this thesis is to characterize the intrinsic properties of the Rho family members and their regulatory proteins: Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

1.1 Rho family proteins

The Rho (*Ras homolog*) family is an integral part of the Ras superfamily of guanine nucleotide-binding proteins (GNBPs) and function as molecular switch of cell by cycling between a resting inactive GDP-bound state and active GTP-bound state. There are at least 22 Rho genes exist in human genome encoding 25 Rho proteins. Rho proteins are highly conserved throughout the evolution from yeast to human. They are synthesized in cytosol and become associated with inner side of plasma membrane after posttranslational modification, where they function as crucial signal transmitters and participate as central control elements in signal transduction that touch on virtually each and every aspect of cellular processes. Rho family proteins are important for many reasons: (i) An approximately 1% of human genome encodes proteins that either regulate or are regulated by direct interaction with Rho family proteins, (ii) They control fundamental cellular processes in all eukaryotes, including cell morphogenesis, polarity, movement, cell division, gene expression, cytoskeleton reorganization and vesicular trafficking (Wennerberg and Der, 2004; Jaffe and Hall, 2005), and (iii) They are associated with a series of human diseases (Ellenbroek and Collard, 2007).

The signature biochemical properties of Rho proteins include binding and exchange of guanine nucleotides (GTP or GDP) and the hydrolysis of GTP. The Rho family proteins are GTP hydrolyzing enzymes (GTPases) and contain at least one conserved GTPase (G) domain located in the N-terminus. They contain a C-terminal hypervariable region (HVR), which is mostly modified by one or several lipid groups. In this study, we focus on the G-domain of Rho family proteins. Although the members of

Rho family are highly conserved according to their amino acid sequences, they remarkably differ in their intrinsic signature biochemical properties (Jaiswal et al., 2012c).

1.1.1 Gene organization and evolutionary history

It is now 26 years since the first member of the Rho family, *rho* gene, was identified by Richard Axel's group in the mollusc *Aplysia* during the search of *ras* related genes and subsequently it was discovered in mammals (Madaule and Axel, 1985). Four years later, the *rac1* and *rac2* genes were isolated from differentiated human leukemia cell line cDNA library (Didsbury et al., 1989). The *cdc42* gene was originally identified in *Saccharomyces cerevisiae* as temperature sensitive cell-division-cycle mutant (Adams et al., 1990). In the years following many other *rho* gene have been identified in eukaryotic kingdom as they are absent in eubacteria and archea. Whereas few eukaryotes lack *rho* genes, a majority of eukaryotic species carry multiple Rho paralogs and they have been identified in *S. cerevisiae* (fungi), *Arabidopsis thaliana* and *Oryza sativa* (plants), *Dictyostelium discoideum* (mycetozoans), *Drosophila melanogaster*, *Caenorhabditis elegans* and in *Homo sapiens* (Foster et al., 1996; Ridley, 1996; Tanaka and Takai, 1998; Valster et al., 2000; Rivero et al., 2001; Venter et al., 2001; Wherlock and Mellor, 2002; Wennerberg and Der, 2004; Jaffe and Hall, 2005; Boureux et al., 2007). A study by Boureux et al. has elucidated the evolutionary history for Rho family over 20 species covering major eukaryotic group from unicellular organism to mammals and redefine the chronology of the emergence of the different subfamilies of Rho family (Boureux et al., 2007). It revealed the fact that Rac is the founder of whole Rho family. Rho, Cdc42, Wrch1/Chp1, and RhoBTB subfamilies appeared before Coelomates and TC10/TCL, Cdc42 isoforms, RhoD/Rif, and Rnd emerged in chordates. Rac1b emerged in amniotes while RhoD only in therians (mammals) (Boureux et al., 2007). This wide distribution of Rho proteins from lower eukaryotes to mammals underlies their importance in eukaryotic cell biology.

On the basis of sequence homology human Rho family can be divided into eight subfamilies: (1) Rho (RhoA, RhoB, RhoC); (2) Rac (Rac1, Rac1b, Rac2, Rac3, RhoG), (3) Cdc42 (Cdc42, G25K, TC10, TCL (TC10-like), Wrch1, Chp1); (4) RhoD (RhoD, Rif); (5) Rnd (Rnd1, Rnd2, Rnd3); (6) TTF/RhoH; (7) RhoBTB (RoBTB1, RhoBTB2) and (8) Miro (Miro1 and Miro2) (Fig. 1), (Wennerberg and Der, 2004; Boureux et al., 2007; Jaiswal et al., 2012c). On the basis of their functional properties they can be

divided into two classes (Jaiswal et al., 2012c). A conventional class includes RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, RhoG, Cdc42, G25K, TC10, TCL (TC10-like), while an non-conventional class includes Rnd1, Rnd2, Rnd3, Rac1b, TTF/RhoH, Wrch1, Chp1, RhoD, Rif, RoBTB1, RhoBTB2, RhoBTB3, Miro1 and Miro2 (Fig. 3).

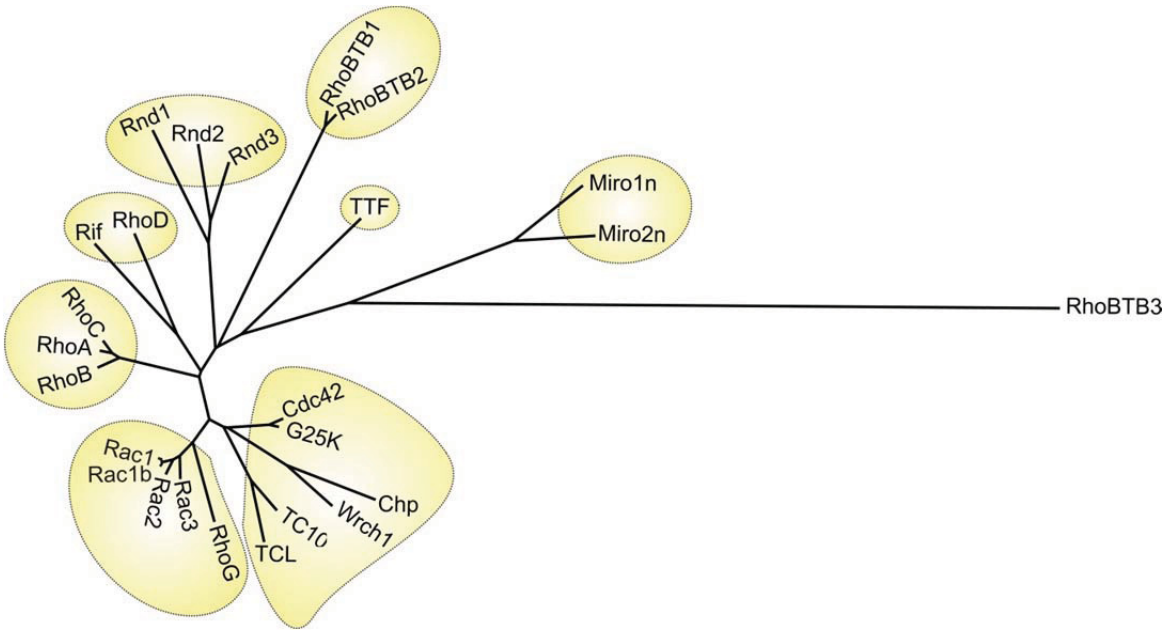

Figure 1. The phylogenetic tree depicting the relationship between Rho proteins
The dendogram demonstrate the relation between 25 members of Rho family. Surrounded shapes indicate the eight subfamilies: RhoA-like, Rac1-like, Cdc42-like, Rnd-like, RhoD-like, TTF, Miro-like and RhoBTB-like. Sequence analysis shows that the G-domain of RhoBTB3 is poorly conserved and do not possess much similarity to other RhoBTB family members 1 and 2, indicating that RhoBTB3 can be excluded from typical Rho family. Espinosa et al. have shown that the RhoBTB3 binds and hydrolyzes ATP rather than GTP (Espinosa et al., 2009). The tree was constructed using full length sequences of the human proteins with the application of MUSCLE multiple sequence alignment tool (Edgar, 2004) and the MEGA software (http://www.megasoftware.net/).

1.1.2. Role in cellular processes

The fact that the members of Rho family are highly conserved throughout the evolution from lower eukaryotes to mammals strongly suggests that they have essential cellular function. And indeed, Rho family proteins have been implicated in the regulation of a broad spectrum of cellular processes. The best characterized function of Rho family proteins is the regulation of actin cytoskeleton (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). The Rho family members later have been shown also to participate in many other pathways that affect cell morphology, proliferation, apoptosis, adhesion (Nobes and Hall, 1995; Allen et al., 1997), polarity (Johnson, 1999), motility (Keely et al., 1997), differentiation, gene expression, and vesicular trafficking (Erickson et al., 1996; Musch et

al., 2001), actin reorganization (Ridley and Hall, 1992; Allen et al., 1998; Ridley, 2001a, 2001b).

The regulation of Rho proteins is mediated by integrated internal signaling and/or extracellular signaling from numerous receptors including G-protein coupled receptors (GPCRs) (Chiariello et al., 2010), growth factor receptors (Hall, 1998), cell adhesion receptors such as integrins (Price et al., 1998; DeMali et al., 2003), cadherins (Braga, 2002) and immunoglobulin superfamily members (Thompson et al., 2002). The Rho family members regulate actin dynamics by acting as molecular switches that transduce signals from activated membrane receptors to cytoskeleton organizers (Van Aelst and D'Souza-Schorey, 1997). Unique changes of filamentous actin (F-actin) are associated with the activation of individual Rho proteins. Briefly, activated Cdc42 induces the formation of microspikes or filopodia, thin finger-like extensions containing F-actin bundles and probably involved in the recognition of the extracellular environment. Rac regulates the formation of lamellipodia or ruffles, curtain-like extensions often formed along the edge of the cell. Rho mediates the formation of stress fibers, elongated actin bundles that traverse the cells and promote cell attachment to the extracellular matrix through focal adhesions (Nobes and Hall, 1995). These specific changes of F-actin have been widely used as markers of the activation of individual Rho family members. In addition to stress fibers, lamellipodia, and filopodia, F-actin can also be arranged into peculiar dot-like structures called podosomes which are controlled by a combination of Rho protein activities (Linder et al., 1999; Chellaiah et al., 2000; Burns et al., 2001).

1.1.3. The role of Rho family proteins in human disease development

Since Rho proteins are involved in various cellular processes ranging from cytoskeleton remodeling and gene expression to cell proliferation and membrane trafficking, it shows that the regulation of Rho proteins is critical for physiological integration of cell. Therefore deregulation or dysfunction of Rho-regulated signaling pathways by any means either loss-of-function or constitutive gain-of-function mutations can contribute to disturbed cellular phenotypes and lead to severe human diseases, such as cancer, mental retardation and immunological disorders (Ambruso et al., 2000; Sahai and Marshall, 2002; Nadif Kasri and Van Aelst, 2008).

In contrast to Ras proteins, which are frequently mutated in many types of human tumors (Klockow et al., 2000; Gremer et al., 2008), no oncogenic mutations have been

found in Rho proteins yet. RhoH/TTF is the only exception of Rho proteins so far to be a putative case of lymphoma development (Dallery et al., 1995; Preudhomme et al., 2000) by rearrangement of the RhoH gene. RhoH specifically express in hematopoietic cells. Despite RhoH/TTF being the only example of a Rho-specific mutation in humans thus far, it has been clearly demonstrated that Rho-family members play an important role in Ras induced transformation.

Surprisingly, overexpression of Rho family members is a far more common occurrence in tumor tissues (Fritz et al., 1999; Forget et al., 2002), such as elevated levels of RhoC observed in metastatic melanoma and gastric carcinomas (Clark et al., 2000), RhoA in breast and testicular cancers (Simpson et al., 2004), Rac1 and Rac3 in prostate and breast cancers (Engers et al., 2007) and the splice variant Rac1b in colorectal and breast cancers (Jordan et al., 1999). Altered Rho GTPase activity or expression is also implicated in cancer progression (Ellenbroek and Collard, 2007; Vega and Ridley, 2008). Alterations in many Rho proteins-dependent cellular functions are found during the progression of a variety of human diseases including mental retardation (Govek et al., 2005), tumor invasion and metastasis (Schmitz et al., 2000; Boettner and Van Aelst, 2002; Jaffe and Hall, 2002; Sahai and Marshall, 2002). Recently, altered distribution of RhoA was found in Alzheimer's disease transgenic mouse, diseased human brain and mice overexpressing amyloid beta protein precursor (AbetaPP) (Huesa et al., 2010). However, there is also example of Rho protein RhoBTB2 that exerts tumor suppressor effects. RhoBTB2 was identified independently as a gene deleted in breast tumors and shoed inhibitory activity when reintroduced into RhoBTB2-defeicient cells (Hamaguchi et al., 2002). There is much supporting functional experimental evidence available for a role of Rho GTPases and their respective GEFs in cancer, particularly in cell motility and invasion through their influence on the actin cytoskeleton (Olson and Sahai, 2009).

1.1.4. Structural features of Rho family proteins

Rho family proteins are approximately 20-25 kDa in size and on the basis of their primary sequence can be divided in three structural parts: the N-terminal region called G-domain, allows Rho family proteins to bind with guanine nucleotides (GDP/GTP), the C-terminal HVR, allows membrane anchorage, and the Caax-motif, a site for posttranslational modifications (Fig. 2, 3).

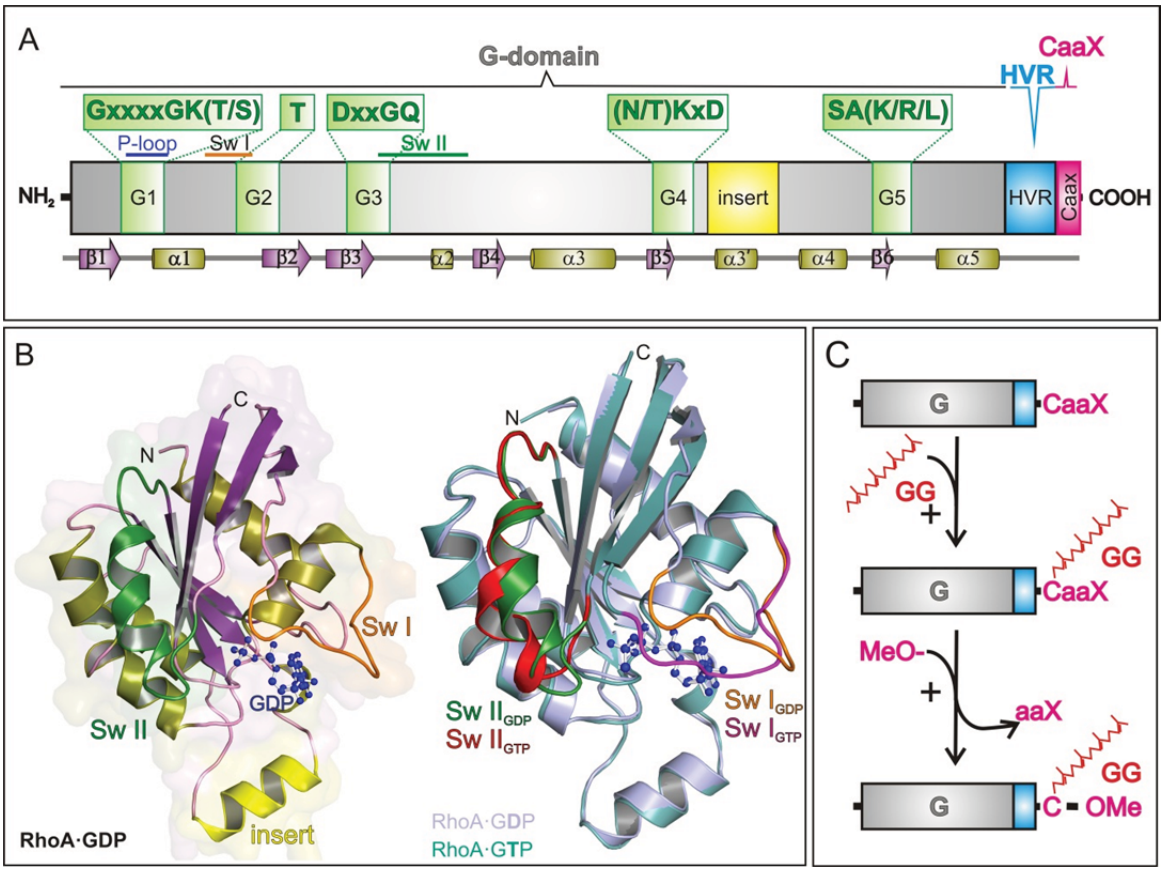

Figure 2. Structural features of Rho family
A) Primary structure. Schematic drawing of a typical Rho protein with G domain (grey), Rho insert (yellow), C-terminal hypervaraible region (HVR: blue) and the Caax-motif (pink). The five conserved G-box motifs (G1-G5 in green) for guanine nucleotide binding and GTPase activities are shown above with their consensus sequence which is based on comparison of 15 Rho proteins (Jaiswal et al., 2012b). Secondary structural elements (α-helices: olive cylinders, β-strands: purple arrows) have been represented at the bottom. B) Tertiary structure of G-domain. The three-dimensional structure of Rho proteins represented as cartoon on RhoA structure (PDB:1FTN) (in left). The structural alignment of Rho·GDP (PDB:1FTN) and Rho·GTP (PDB:1A2B), represented as cartoon (in right), shows the shift of switch regions upon nucleotide binding. The formation of the GTP-bound state and GDP-bound state of the Rho is accompanied by conformational changes mainly at these two regions: switch I and II. C) Posttranslational modifications of Rho family. The Rho family proteins are prenylated on the cysteine in the CaaX-motif at C-terminal region. This modification is followed by proteolysis of last three amino acids and methylation on the terminal cysteine. Letters for amino acids: A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; K, Lys; N, Asn; S, Ser; x, any amino acid.

1.1.4.1. G-domain

The G domain is a minimal region of Rho proteins necessary for guanine nucleotide binding and hydrolysis. All members of Rho family share high similarity within G-domain. The conserved G domain has an approximate molecular mass of 20 kDa with a typical fold consist of five α-helices (α1-α5), six beta-sheets (β1-β6) (Fig. 2A, B). It consists of five conserved sequence motifs, called "G box" (G1 to G5 box) around the guanine nucleotide binding site (Fig. 2A) (Saraste et al., 1990; Bourne et al., 1991; Vetter and Wittinghofer, 2001). These five conserved motifs, which together constitutes

G domain, have conserved structure and function in all Rho family members. The G1 box also known as P-loop (phosphate binding loop; residues 12-20 Rho numbering) with the consensus sequence aaaaGxxxxGK(T/S), where a = C, V, T, L, I, or M, and x = any amino acid, (Saraste et al., 1990), originally termed the Walker A motif (Walker et al., 1982) and interacts with the α- and β-phosphates of the nucleotide. The G2 box (residues 34-42 Rho numbering) has only threonine (Thr-37 RhoA numbering) almost invariable and is involved in magnesium ion (Mg^{2+}) coordination and direct binding to the γ-phosphate (Fig. 2A). The G3 box (residues 55-63 RhoA numbering), with the structure blbbDxxGQ (b = hydrophobic, and l = hydrophilic), is involved in binding a nucleotide-associated Mg^{2+} ion (Fig. 2A). The conserved aspartate (Asp-59 RhoA numbering) binds to the Mg^{2+} via a water molecule and the glycine (Gly-62 RhoA numbering) makes a main chain contact to the γ-phosphate. Together these two G2 and G3 motifs, which are the integral elements of the switch I and II regions, trigger conformational changes when the γ-phosphate of GTP is hydrolyzed. The switch regions are the main determinants for the interaction of regulators and effector molecules (Dvorsky and Ahmadian, 2004). The G4 box (residues 113-121 RhoA numbering) (bbbb(N/T)KxD) makes contact with the guanine ring through hydrogen bond and is the major determinant of guanine base-binding specificity (Fig. 2A). The aspartate (Asp-120 Rho numbering) makes a bi-furcated contact to the guanine ring ensuring the specificity. The G5 box (residues 157-162 Rho numbering) although important for binding to the guanine base and often with sequence motif SA(K/R/L), is only weakly conserved and is the most divergent motif. It makes indirect associations with the guanine nucleotide (Fig. 2A). The alanine (Ala-161 Rho numbering) of G5 motif makes a main chain interaction with the guanine base (Bourne et al., 1991; Jiang and Ramachandran, 2006; Wittinghofer and Vetter, 2011). RhoH/TTF and Rnd 1/2/3 proteins of Rho family do not contain conserved Gly and Gln at position 14 and 63 (RhoA numbering), respectively, hence appear to constitutively bound to GTP (Bishop and Hall, 2000; Fiegen et al., 2002; Jaiswal et al., 2012c).

1.1.4.2. Hypervaribale region (HVR)

Like Ras, the Rho family proteins contain a C-terminal a HVR (important for membrane localization) ending with a CaaX motif (Fig. 2, 3). Most of the Rho family

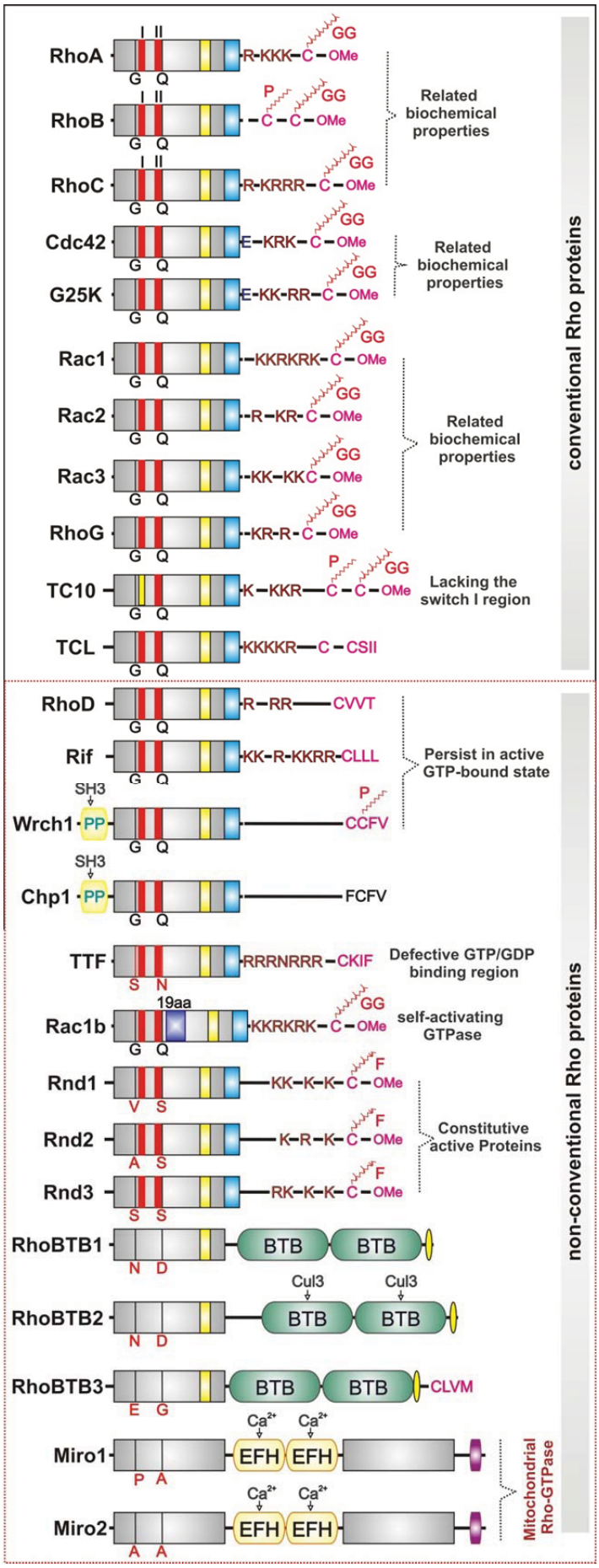

Figure 3. Schematic overview of domain organization and biochemical properties of Rho family proteins
Domain organization of 25 members of Rho family shows that RhA/B/C, Rac1/2/3, RhoG, Cdc42, G25K, TC10, TCL, RhoD and Rif have similar basic structure. Wrch1 and Chp1 are characterized by the presence of N-terminal proline-rich region. Rnd1/2/3, and TTF are considered as atypical Rho proteins as they do not have essential catalytic amino acids, including Gly-14 (RhoA numbering) and Gln-63 (RhoA numbering) that make them lack of GTPase activity. Rac1b has additional 19-amino acid insertion next to the switch II region, which make it self-activating Rho protein. RhoBTB and Miro proteins have most divergent domain organization. RhoBTB proteins contain additional to G-domain two BTB domains and NLS, nuclear localization sequence. Although RhoBTB3 possess C-terminal Caax motif, the G-domain of RhoBTB3 is poorly conserved and do not possess much similarity to other RhoBTB family members 1 and 2. Miro proteins are mitochondrial RhoGTPases. Both, Miro1 and Miro2 contain two G domains, in which only the N-terminal G domain have certain similarity to typical Rho proteins. Miro proteins neither have the Rho insert helix nor the C-terminal CaaX-motif, which are the characteristic features of Rho family proteins. In addition to two G-domains Miro1 and 2 proteins possess two EFH domains (EF-hands, for calcium binding) and one TM (transmembrane) domain. The Rho family proteins are prenylated on the cysteine in the CaaX-motif at C-terminal region. Examples of possible prenylations are: geranyl-geranylation (GG), farnesylation (F) and palmotylation (P). Most Rho proteins terminate in a motif (X = L) that dictates geranylgeranylation of the cysteine residues of the CaaX motif. Exceptions include the Rnd subgroup, which undergo farnesylation, and RhoB, which has two populations of lipid modified protein. Wrch-1 has been shown to be palmolyted. Interestingly, Chp does not have a CaaX motif and, instead, terminates in a FCFV sequence, making it an unlikely candidate for isoprenylation.

members, except for Chp1, RhoBTB1, RhoBTB2 and Miro1 and 2, have CaaX motif consisting of a cysteine (C), two aliphatic residues (aa) and a variable amino acid (X) (Fig. 3). The cysteine of the CaaX motif serves as a substrate for isoprenylation by prenyltransferase enzymes such as farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) (Fig. 2C) (Casey and Seabra, 1996). The protein is a substrate for GGTase I if the X residue of the CaaX motif is an L or F residue (in Cdc42, Rac1/2/3 and RhoA/C). However, CaaX motifs terminating in S, M, Q, T, I or A (in Rnd1/2/3, TC10, TCL) are usually substrates for FTase (Cox, 1995; Wennerberg and Der, 2004) (Fig. 2B). Interestingly, membrane targeting of Wrch1 is distinct to the classical CaaX motif-containing Rho GTPases and has been reported to be achieved by palmitoylation of a CFV motif in its C-terminus (Berzat et al., 2005). This posttranslational modification on a conserved cysteine residue of the CaaX-box is responsible for the subcellular localization of Rho proteins between cytosol and plasma membrane, their site of action. Incorporation of farnesyl or geranyl-geranyl group is required for proper membrane anchoring and biological activity of Rho proteins. Interestingly, RhoB can be either farnesylated or geranyl-geranylated and these modifications localize the protein either in the plasma membrane or in endomembranes, respectively (Lebowitz et al., 1997). The subcellular localization of Rho proteins is also affected by the polybasic region located just upstream of the Caax-motif (Fig. 3). Small sequence variation in this polybasic region determines intracellular localization and/or biological effects of very homologous Rho proteins, e.g. Rac subfamily where small changes in the polybasic region determines the different intracellular locatization of Rac1, Rac2, Rac3 and RhoG (Prieto-Sanchez and Bustelo, 2003; Hajdo-Milasinovic et al., 2007).

A unique structural feature of the Rho family proteins is a α-helical “insert region” which is characterized by an up to 13-amino acid sequence, located between the β5 and α4 (Fig. 2A, B) (Valencia et al., 1991). Rho-insert is required for interaction of Rho proteins with its effector molecules.

1.1.5. GTPase cycle: Classical biochemical model for Rho proteins

The signature biochemical properties of Rho proteins include binding, exchange and hydrolysis to the guanine nucleotides and they share a common biochemical mechanism like other Ras family members (Dvorsky and Ahmadian, 2004). They act as

binary molecular switches by cycling between GTP-bound to GDP-bound form like other members of Ras superfamily (Fig. 4) (Van Aelst and D'Souza-Schorey, 1997). In the GTP-bound (ON) form Rho proteins acquire the active conformation and thereby able to interact with its effector molecules and activate downstream effectors leading to a variety of signaling cascades while GDP-bound form (OFF or inactive conformation) switch off the signals. The amount of GTP in cell is supposed to be much higher than the amount of free GDP therefore the current model for Rho protein function predicts that Rho proteins exist in equilibrium between active and inactive state. The state of this equilibrium is the result of the interaction of intrinsic biochemical parameters of the Rho protein with external factors. Intrinsic parameters are the concentration of Rho proteins, their relative affinities for GTP and GDP, the GTP/GDP exchange rate, the intrinsic GTPase activity and other conformational changes caused specific mutations that may affect the intrinsic biochemical properties (Jaiswal et al., 2012c). External factors include regulatory proteins GEFs, GAPs and GDI, Mg^{2+} concentration.

Although the involvement of a guanine nucleotide is a prerequisite for members of the Rho family, GTPase activity itself is not, as members of the Rnd and TTF/RhoH subfamilies are GTPase deficient (Nobes et al., 1998; Li et al., 2002). RhoE/Rnd3(Foster et al., 1996; Fiegen et al., 2002; Wennerberg et al., 2003), Rac1b (Fiegen et al., 2004) have been shown to exist in a constitutively GTP-bound form. This is one of several examples of the diversity of the proteins classified as Rho family. To this we can also now include Wrch (Shutes et al., 2006), RhoD and Rif (Jaiswal et al., 2012c) Rho family members, as these members also found to stay as GTP bound state in resting state of cell.

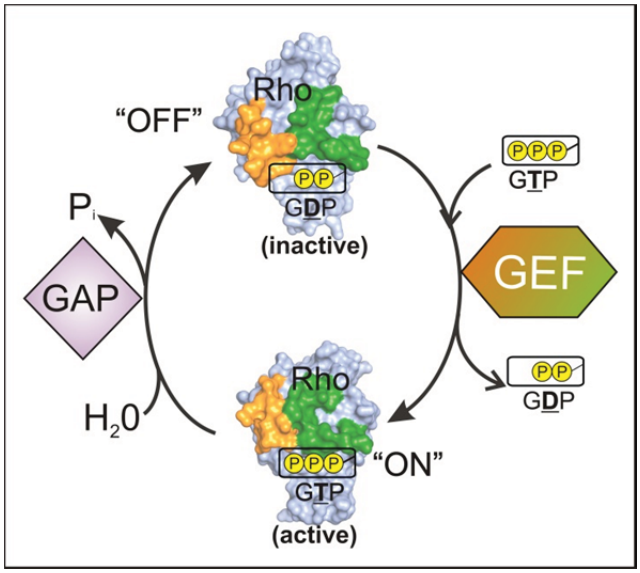

Figure 4. The GTPase regulatory cycle

The Rho proteins act as molecular switches that cycle between an inactive GDP-bound state and active GTP-bound state. The intrinsic nucleotide exchange activity is catalyzed by a guanine nucleotide exchange factor (GEF) while intrinsic GTPase reaction is catalyzed by a GTPase-activating protein (GAP). Switch regions that change conformation based on the state of bound nucleotide are depicted in green (Sw I) and orange (Sw II). For sake of simplicity, intermediate steps in the regulatory cycle have been omitted.

1.2. Regulation of the Rho family GTPases

1.2.1. Control of the molecular switch function

Like other small GTPase, the Rho family proteins also works as binary molecular switch, and their function depends on controlled binding and hydrolysis of GTP, which converts the Rho family proteins between two states: Rho bound to GTP (abbreviated as Rho·G**T**P) and Rho bound to GDP (abbreviated as Rho·G**D**P). Rho·G**T**P referred to as the "active" form or "ON" state. With GTP bound, the Rho protein forms a conformation that allow it to interact with effector molecules that in turn convey a molecular signal for downstream signaling process. In contrast, when GTP is hydrolyzed Rho is bound to GDP, and this conformation considered as the inactive form or "OFF" state (Fig. 4). This process of switching conformations of Rho proteins starts a "molecular clock" of the cell in which the duration of signal depends on the length of time GTP is bound to the Rho proteins because the GTP-bound state determines whether Rho protein is in the "on state". Therefore Rho proteins considered as a molecular switches for the timings and specificity of events that take place within the cell. For most of the Rho family proteins nucleotide affinities are high (Jaiswal et al., 2012c) while nucleotide dissociation rates, and consequently intrinsic (spontaneous) nucleotide exchange, are slow compared with the biological process being controlled. And same is true for GTP hydrolysis rate. Although intrinsic GTP hydrolysis rate of Rho proteins is slow, it is faster than intrinsic nucleotide exchange. This shifts the equilibrium of Rho protein largely towards GDP-bound state. Therefore, to control the duration of signals and so on Rho-dependent biological process, the Rho family proteins critically depend on regulator proteins.

1.2.2. Different classes of Regulators (GEFs, GAPs, GDIs)

In order to control the "molecular switch" and therefore duration of signal, the cell has evolved three main regulatory proteins that function to control the GTPase cycle of the Rho family proteins. (i) Guanine nucleotide exchange factors (GEFs) which stimulate the intrinsic rate of GDP release of Rho family proteins up to 7 orders of magnitude, and the binding of GTP to the Rho proteins which results in the conversion of Rho protein to "on" or "active" state (Fig. 4) and hence initiate the signal cascade (described in detail in section 3). (ii) GTPase activating proteins (GAPs) which terminate the signal by stimulating the hydrolysis of GTP by Rho proteins. GAPs facilitate the hydrolysis of GTP

up to 5 orders of magnitude, and by this transform the Rho proteins into their "off" or "inactive" state (Fig. 4) (described in detail in section 4). (iii) GDP dissociation inhibitors (GDI) which works as passive regulator by simply holding inactive (GDP-bound) form of Rho family proteins in cytosol. The shuttling of Rho proteins between cytosol and the plasma membrane and their activation by GEFs, are controlled by third class of regulatory proteins of Rho family known as GDI (GDP dissociation inhibitors) (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005; Garcia-Mata et al., 2011). GDIs can hide the prenylated C-terminal part of Rho proteins and extract these proteins from the lipid environment of membranes by binding to the hypervraible region of Rho proteins (Michaelson et al., 2001). Another function of GDI was uncovered recently by that RhoGDI are critical for both homeostasis of Rho proteins and cross talk between Rho family members (Boulter et al., 2010).

Beside from this canonical regulation of Rho family proteins by GEFs, GAPs and GDIs, recent findings show additional levels of regulation of Rho proteins such as transcription, posttranslational modification and degradation (Engel et al., 1998). Most of the Rho proteins are ubiquitously expressed but some show tissue or cell specific expression. For instance, expression of Rac2 is restricted to haematopoietic cells only. The expression of RhoG is induced during the G1 phase of the cell cycle (Vincent et al., 1992), whereas Wrch1 is Wnt responsive gene of which the expression is induced by activation of the Wnt signaling pathway (Tao et al., 2001). Additional level of Rho proteins regulation is controlled by local changes in protein stability or degradation. Regulation of Rho proteins via protein degradation is shown through by ubiquitination and subsequent targeting of the proteasome (Visvikis et al., 2010; Doye et al., 2012). Therefore, ubiquitination is now known beyond its simple house-keeping task of tagging Rho proteins for degradation to also regulating the spatio-temporal dynamics of Rho GTPase activity, including an alternative way to terminate GTPase activity (Schaefer et al., 2012). Analysis of inactive forms of Rho proteins have also revealed that some classes of Rho proteins may become inactivated via phosphorylation. for example, RhoA, is phosphorylated by PKA at serine 188, and this phosphorylation has been shown to increase RhoA interactions with GDIs by inhibiting binding of RhoA to other downstream targets independent of RhoA's GDP/GTP state (Forget et al., 2002).

1.3. Gunanine nucleotide exchange factors (GEFs)

Rho GTPases are highly regulated proteins and guanine nucleotide exchange factors (GEFs) are the principle mediators of their activation. GEFs are the upstream activator of Rho family proteins and act as a positive regulator of the GTPase cycle by keeping the switch "ON" (Fig. 4). They achieve this in two ways: by destabilizing GDP-GTPase interaction leading to GDP release, and by stabilizing this nucleotide-depleted transition state, enabling GTP (which is at higher concentration in the cell than GDP) to bind to the GTPase. GEFs are different from other Rho-interacting proteins by their preferential binding to nucleotide free (nf) GTPase compared with the GDP or GTP bound forms (Hart et al., 1996; Cherfils and Chardin, 1999).

1.3.1 Dbl family GEFs

In eukaryotes a diffuse B-cell lymphoma (Dbl) family (Rossman et al., 2005) has been described as conventional RhoGEFs and members of Dbl family are characterized by a unique catalytic Dbl homology (DH) domain, which display accelerated nucleotide exchange activity of Rho family proteins (Fig. 5). Dbl family GEFs are the major class of nucleotide exchange factors and first to be reported as positive regulator for the activation of Rho family proteins. The Dbl family named after its founding member mammalian Dbl protein, which was isolated as an oncogenic product from diffuse B-cell lymphoma cells during an oncogene screen (Srivastava et al., 1986; Eva et al., 1988). The analysis of Dbl protein primary structure enabled finding of a minimal region called as DH domain, which shared a significant similarity with Cdc24 yeast protein. The suspect, that Cdc24 act upstream of Cdc42 led to conclude that both Cdc24 and Dbl proteins function as GEFs for Rho proteins. It was showed for the first time, this minimal region "DH domain" of Dbl protein is necessary for GEF activity by direct activation of Cdc42 (Hart et al., 1991; Hart et al., 1994). The Dbl family consists of 74 members in human (Jaiswal et al., 2012b) with evolutionary conserved orthologs in fly (23 members), yeast (6 members), worm (18 members) (Venter et al., 2001; Schmidt and Hall, 2002a) and slime mold (45 members) (Vlahou and Rivero, 2006). However, till date no DH domain containing proteins have been identified in plants (Schmidt and Hall, 2002a). Dbl family proteins are multimodular proteins with DH domain as signature of the Dbl family. Almost all Dbl family proteins have a PH domain adjacent to C-terminal to their DH

domain. PH domain has been proposed to functions in localization to the plasma membrane, as PH domains are known to bind to both phosphorylated phosphoinositides (PIPs) and proteins (Rebecchi and Scarlata, 1998; Lemmon and Ferguson, 2000). Apart from their hallmark DH-PH cassette, Dbl family proteins are highly divergent and contain additional domains with diverse functions as SH2, SH3, CH, RGS, PDZ, IQ domains which responsible for interaction to proteins or FYVE, C1, C2 domains for interaction to lipids and other domains like Ser/Thr kinase, RasGEF, RhoGAP, RanGEF, or additional PH domains (Fig. 5) (Schmidt and Hall, 2002a) which can play a role in autoregulation, subcellular localization and connection to upstream signals (Rossman et al., 2005; Garcia-Mata and Burridge, 2007).

Apart from conventional Dbl family GEFs some non-conventional or atypical GEFs also have been described which activate Rho proteins. These atypical GEFs share no sequence or structural homology with the conserved regions of the catalytic DH domain of Dbl family proteins (Fig. 6) and likely utilize novel means to engage Rho GTPases. Additionally, these various atypical RhoGEFs lack any overall sequence or structural similarity with each other. These candidate atypical GEFs for Rho proteins described in literature include: (i) CZH (CDM-zizimin homology) family GEFs (Meller et al., 2005). The CZH family include: Dock180 and zizimin proteins, which activate Cdc42. DOCK (Dedicator of cytokinesis) family RhoGEFs are characterized by two regions of high sequence conservation that are designated DHR1 (Dock-homology region regulatory) and catalytic (DHR2) domains. The CZH (CDM-zizimin homology) activate Rho proteins through DHR2 (DOCK (dedicator of cytokinesis) homology region 2) domain. DOCK180 displays Rac GEF activity which has been mapped to a region called the ‘Docker/Dedicator of cytokinesis’ domain (Brugnera et al., 2002) and it requires a cofactor protein (ELMO) for its function (Brugnera et al., 2002). (ii) SopE/WxxxE-type exchange factors, that are classified as type III effector proteins of pathogenic bacteria (Bulgin et al., 2010). (iii) RopGEFs which activate Rho proteins from plants (called Rops) (Berken et al., 2005). RopGEFs activate Rops by their catalytic domain called PRONE (plant specific Rop nucleotide exchange factors). It has been observed that some human Dbl GEFs are capable of activating some Rops (plant Rhos) however, the opposite has not been probed (Berken et al., 2005). (iv) Phospholipid binding protein SWAP70 (switch-associated protein 70). One more protein DEF6 (also known as SLAT) has been describes as a novel class of GEFs for Rho proteins and contain a PH but no DH domain. Both SWAP70 and DEF6 are unique as they hold PH-DHL cassette. Both of them have

EF-hand motif at N-terminus, PH domain at center and the region which show very little homology to classical DH domain, of Dbl family at C-terminus, referred as DH-like (DHL) domain. The region between EF-hand motif and PH domain is referred as to EF6-SWAP70 homology (DSH) domain (Mavrakis et al., 2004; Tybulewicz and Henderson, 2009; Biswas et al., 2010).

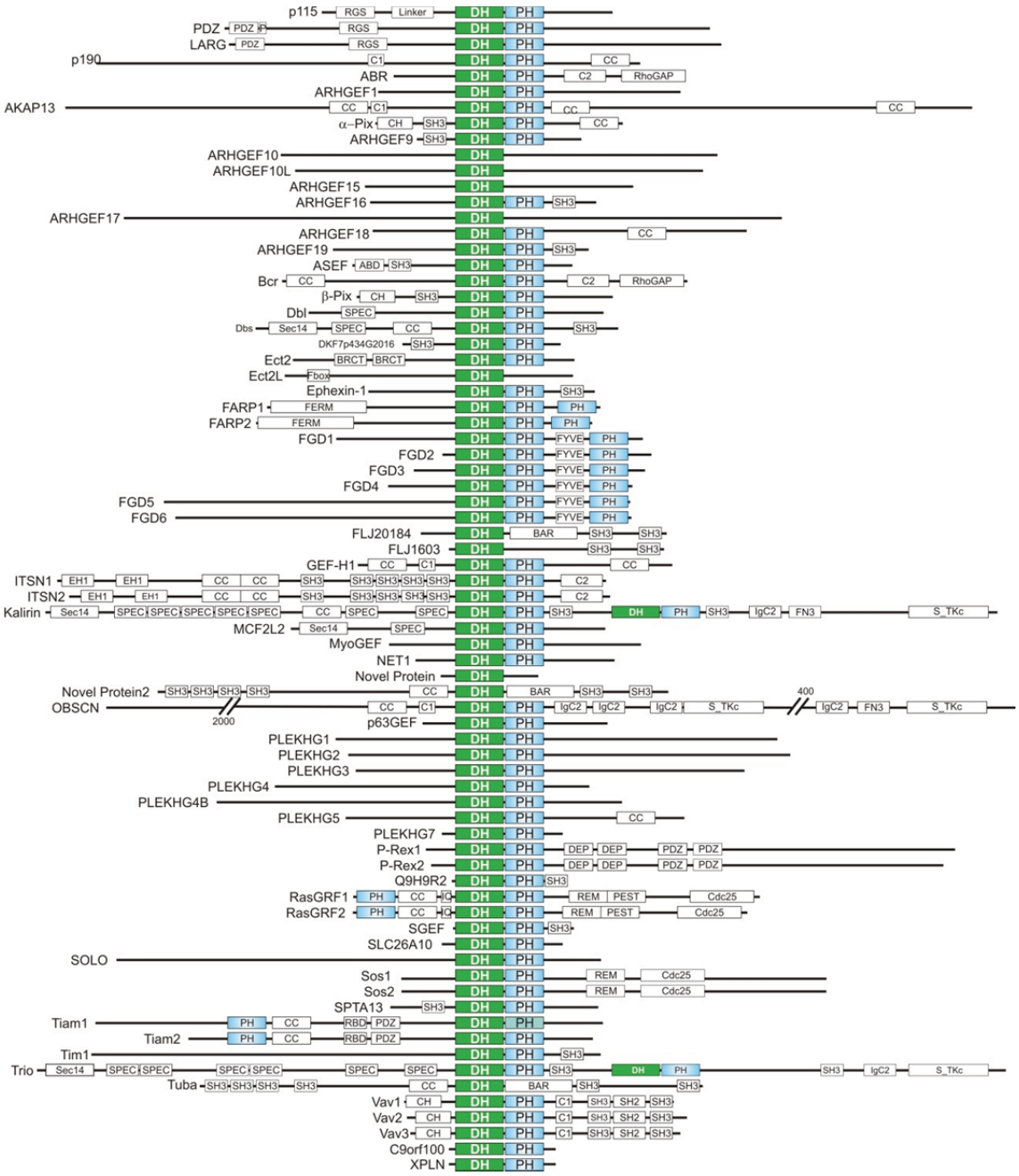

Figure 5. Schematic overview of domain organization of Dbl family proteins
The schematic representation of the domain organnization of Dbl family GEFs are illustrated approximately to scale. The catalytic DH domain of Dbl family GEF is highlighted in green. DH domains almost always occur together with a PH domain (light blue) at C-terminal. Some Dbl proteins contain two DH-PH cassette while some Dbl proteins lack tandem PH domain. Abbreviations used for various other functional domains.

Compared to typical Dbl family GEFs (Fig. 5), DEF6 and SWAP70 have an atypical domain arrangement in which PH domain C-terminal linked with DHL Domain (PHDHL) (Fig.6). DEF6 was discovered from mouse haemopoietic tissues (Hotfilder et al., 1999) and SWAP70 was isolated from B-cell nucleus complex (Borggrefe et al., 1998). DEF6 is mainly expressed in T cell whereas SWAP70 is mainly expressed in B cell. DEF6 was shown to to activate Rac1, cdc42 and RhoA while SWAP70 was shown to activate Rac1 and Rho. Both can induce actin cytoskeleton rearrangement and membrane ruffling through PI3K dependent signaling pathway (Shinohara et al., 2002; Mavrakis et al., 2004; Ocana-Morgner et al., 2009). Recently another class of GEFs also has been discovered by Gomez-Cambronero group, called PLD2 (phospholipase D2) activating Rac2 protein having PH domain, but neither DH nor DHR2 domain (Gomez-Cambronero, 2012).

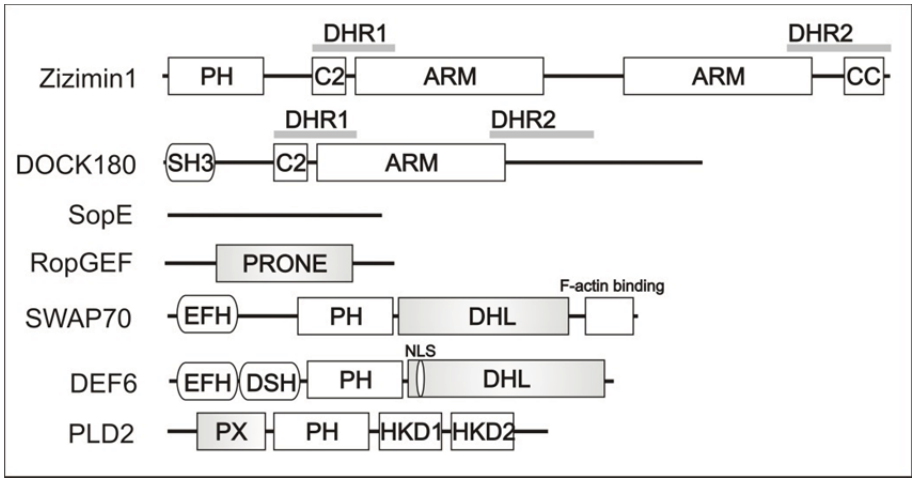

Figure 6. Atypical guanine nucleotide exchange factors for Rho family
The domain organization are shown for atypical RhoGEFs, which lack the canonical DH-PH cassette defined by the Dbl family. Zizimin1 and Dock180 are members of the CZH family, defined by the presence of DHR1 and DHR2 domains essential for guanine nucleotide exchange. Dock180 requires the accessory protein ELMO to function, while Zizimin1 most likely functions as a homodimer. RopGEFs activate plant Rho proteins by their catalytic domain called PRONE. SopE is a bacterial toxin which highjacks the cytoskeletal machinery of host cells during invasion, while SWAP-70 is not well characterized. DEF6 and SWAP70 the region at the C-terminus which shows limited homology to DH domain of classical GEFs is referred to as DH-like (DHL) domain. PLD2 has both a lipase and a GEF activity. Both PX and PH protein domains bind to the Rac.

1.3.1.1. Structural and functional characteristic of DH domain

DH domain is the signature of Dbl family proteins. The catalytic guanine nucleotide exchange activity of Dbl family proteins reside entirely with the DH domain and DH domain is not only sufficient for the catalytic activity but also responsible for the

substrate specificity (Jaiswal et al., 2011; Jaiswal et al., 2012b). The catalytic DH domain consist of approximately 200 residues and from crystallographic and NMR analysis of the DH domain of several Dbl proteins reveals that in three-dimensional structure it composed of unique extended bundle of 10-15 alpha helices (Aghazadeh et al., 1998; Liu et al., 1998; Worthylake et al., 2000). This helical fold is mainly composed of three conserved regions: CR1, CR2 and CR3, each of them 10-30 residues long form separate alpha helices that pack together (Fig. 7) (Hoffman and Cerione, 2002; Erickson and Cerione, 2004). The CR1 and CR3 regions are solvent exposed until complexed with Rho proteins where they along with conserved region of α6 helix then form interaction pocket and make primary interacting contacts with the switch I, β2-β3 and switch II of Rho proteins (Fig. 2 and 5 of Jaiswal et al., 2012b) . Beside these three conserved regions (CR1, CR2 and CR3), DH domains of Dbl family share little homology with each other.

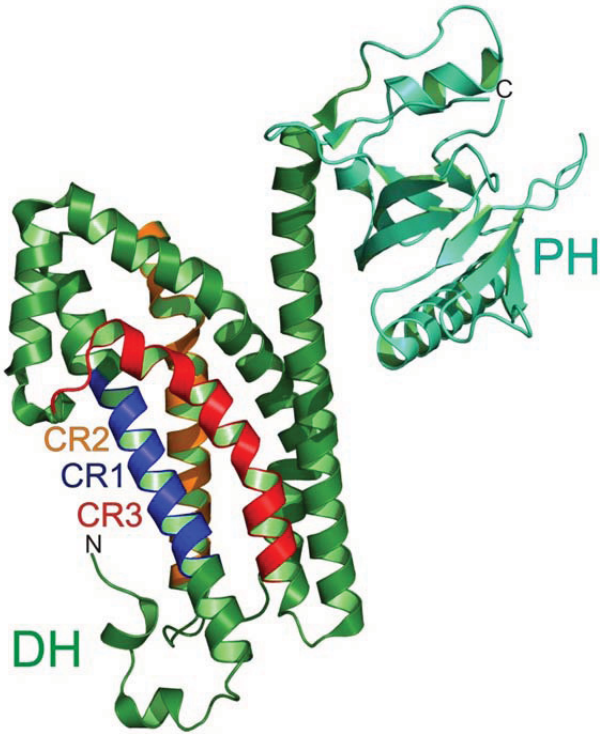

Figure 7. Structural features of DH-PH domain
The DH (left, green) and PH domains (right, teal) of LARG (PDB:1X86) have been represent in cartoon. The three conserved regions CR1, CR2 and CR3 are colored in blue, orange and red respectively.

1.3.1.2. Tandem PH domain of Dbl proteins

In the majority of Dbl family proteins, the catalytic DH domain is preceded by a pleckstrin homology (PH) (Fig. 5) domain of around 100 residues and even the identity between PH domain of Dbl family is less 20%, they share similar three-dimensional

structure with two orthogonal antiparallel β-sheets and the C-terminal α-helix folds in to cover one end (Lemmon et al., 2002). PH domain was originally identified in number of cytoplasmic signaling proteins that display homology to a region repeated in the protein pleckstrin (Tyers et al., 1988; Tyers et al., 1989; Haslam et al., 1993). Together with PH domain Dbl family comprises DH-PH cassette as signature motif of this family. This exclusive coupling of DH-PH cassette in Dbl family proteins indicate an essential and conserved function for PH domain (Hoffman and Cerione, 2002; Rossman and Sondek, 2005; Aittaleb et al., 2010; Jaiswal et al., 2011; Viaud et al., 2012). However, a universal role of the DH domain associated PH domain has not yet been clear. Our current view on the function of PH domain stands as a "membrane targeting device" on the point that they are involved in the recruitment of proteins to the plasma membranes, by virtue of their ability to bind phosphoinositides (Lemmon et al., 1996; DiNitto and Lambright, 2006). This function is supported by the finding that PH domain function as membrane targeting signal in some Dbl family GEFs such as Lymphoid blast crisis (Lbc)'s first cousin (Lfc) (Whitehead et al., 1995). However, the role of DH domain associated PH domains in Dbl family proteins remains controversial, as they have also been reported to participate in direct engagement of Rho proteins and assisting in gunanine nucleotide exchange. This PH domain assisted nucleotide exchange has been well characterized for two Dbl family proteins: Dbs (Rossman et al., 2002) and TrioN (Liu et al., 1998; Chhatriwala et al., 2007). In contrast, inhibition of nucleotide exchange activity via PH domain by masking access to the DH domain in several Dbl proteins including, Vav and Son of Sevenless 1 (Sos1) (Han et al., 1998; Nimnual et al., 1998) have been shown. PH domains of Dbs (Dbl's big sister) (Cheng et al., 2004), Vav2, Tiam1 (T-cell lymphoma invasion and metastasis factor-1) (Arthur et al., 2004), and scambio (Curtis et al., 2004) also have been shown to be effector sites for Ras GTPases. Apart from its membrane-targeting properties, emerging evidence suggests that PH domains may also play important regulatory roles by serving as protein-protein interaction modules (Lemmon, 2004). The PH domain of non-Dbl family proteins, such as G protein receptor kinase 2 (GRK2) (Lodowski et al., 2003), insulin receptor substrate-1 (IRS-1) (Farhang-Fallah et al., 2002) and phospholipase C-β (PLCβ) (Philip et al., 2002) has been previously characterized for mediating protein-protein. Furthermore, some Dbl family proteins such as T-lymphoma invasion and metastasis inducing protein 1 (Tiam1) and RasGRF contain a second (N-terminal) PH domain, and this second PH domain is required for membrane localization

rather than the PH of the DH-PH module (Buchsbaum et al., 1996; Michiels et al., 1997; Stam et al., 1997).

By searching for DH domain containing proteins in the human genome 74 Dbl proteins have been identified (Jaiswal et al., 2012b) (Fig. 5). Interestingly, 9 of them lack the C-terminal tandem PH domain, from which 3 contain a membrane bending and tubulating BAR (Bin/amphiphysin/Rvs) domain (Jaiswal et al., 2012b). In this instance, it has been speculated that the BAR domain can functionally replace the PH domain, due to its membrane-associates capacity (Rossman et al., 2005). In all together, the majority of the data suggests that the PH domain serves to fine-tune the activity of the DH domain in the cell directly be mediating the engagement of regulatory proteins or indirectly by serving as hydrophobic tethers which can improve enzymatic activity and stability of protein in non-aqueous solution.

1.3. 2. Mechanism of GEF induced nucleotide exchange

Common mechanism utilized by GEFs is to deform the phosphate-binding site which results in a reduced affinity of the nucleotide. Up to now, three possible kinetic mechanisms for GEF catalyzed nucleotide exchange have been proposed and the most acceptable common mechanism is allosteric competitive mechanism and involves a series of fast reaction steps, which lead from a binary protein-nucleotide complex Rho·GDP to a ternary Rho·GDP·GEF and finally to a binary nucleotide-free complex Rho·GEF (Cherfils and Chardin, 1999). It has been tested for some Ras superfamily members (Klebe et al., 1995; Lenzen et al., 1998; Goody and Hofmann-Goody, 2002).

According to this, mechanism GEFs first bind to the GDP-bound form of Rho (Rho·GDP·GEF), reducing the affinity of the Rho protein for GDP (or GTP as the case might be) by accelerating dissociation. A nucleotide free Rho protein Rho and GEF complex is formed (Rho·GEF). GEF therefore destabilize the Rho·GDP complex while stabilizing the nucleotide free reaction intermediate (Cherfils and Chardin, 1999). Since the intracellular ratio of GTP:GDP is high, the released GDP is displaced with GTP, GTP binds to the guanine nucleotide pocket of the proteins in complex, forming ternary complex (Rho·GEF·GTP). The complex Rho·GEF·GTP dissociates to form free GEF and Rho·GTP, leading to activation of Rho (Fig. 8).

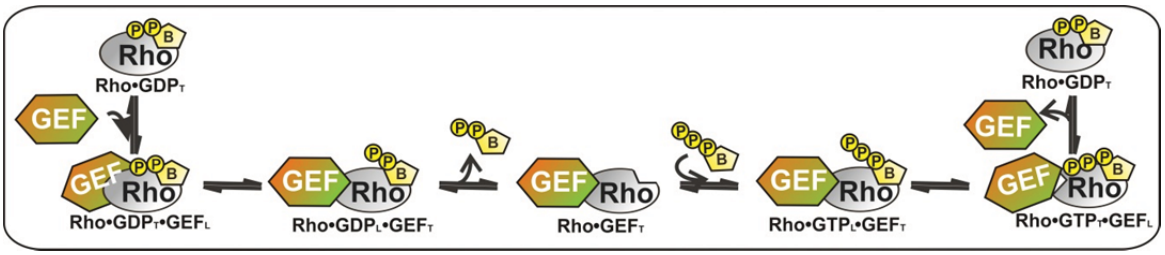

Figure 8. Mechanism of the GEF-catalyzed guanine nucleotide exchange reaction
The nucleotide exchange reaction occurs in successive reversible steps. The GDP/GTP (yellow) interacts with the Rho protein (gray) via its base (B) and its phosphate moieties (P). GEF (green-orange) competes with the GDP nucleotide for binding with the Rho protein and thereby promotes nucleotide exchanges. The competition involves the formation of loose (L) and tight (T) interaction of the Rho protein with the GDP and the GEF. Detail description is in text.

1.3.3. A plethora of Dbl family proteins

It has become evident that Dbl family proteins are more abundant and varies in the cells than Rho family proteins, up to now 74 Dbl proteins against 18 prone substrate Rho proteins have been reported in human (Fig. 3) (Jaiswal et al., 2012b). The reason for 4.1-fold excess of Dbl proteins over Rho proteins is still unclear but it has been suggested that it could be associated to specific signaling and/or cellular requirements needed to activate certain Rho proteins combination/cascade (Rossman et al., 2005; Jaiswal et al., 2012b). Since there are many more Dbl proteins and many of them can activate more than one Rho protein, the activation of Rho proteins promoted by Dbl family proteins constitute a level of regulation in which the signaling pathways can converge or diverge towards one or more Rho protein (Etienne-Manneville and Hall, 2002). The evidence suggests that at least one representative of each Dbl subfamily is expressed in all mammalian cells, but that they may act at distinct subcellular locations. For example, Rac1 is ubiquitously expressed while Rac2 is mainly expressing in hematopoietic cells and Rac3 is primarily expressed in brain, and therefore Rac specific Dbl GEF that can act on all three isoforms of Rac *in vitro* may quantitatively selective and specific for Rac2 if it co-localizes with Rac2 in an intact cell (Jaiswal et al., 2012b).

1.3.4. Regulation and GEF proteins function

The regulation of Dbl family proteins can be as diverse as 74 members of family are multimodular proteins (Fig. 5). For many Dbl family GEFs, N-terminal sequences upstream of the tandem DH-PH domains that catalyze exchange serve as intramolecular, auto-inhibitory sequences. This role is demonstrated by the fact that N-terminal truncations of sequences upstream of the DH-PH domains were responsible for creating the constitutively activated and transforming variants of RhoGEFs identified in transformation or invasion assays. Some Dbl GEFs are activated by phosphorylation at an N-terminal motif that relieves the autoinhibitory activity e.g. Vav (Aghazadeh et al., 2000) and Tim (Yohe et al., 2007). Other mechanisms of activation involve protein interaction with N-terminal domains, such G alpha 12/13 interaction with the RGS box-containing RhoGEFs (p115, Larg and PRG) (Hart et al., 1998; Booden et al., 2002; Suzuki et al., 2009; Jaiswal et al., 2011), Ras interaction with the RBD in Tiam (Lambert et al., 2002) and APC association with the N-terminus of ASEF (Kawasaki et al., 2000; Mitin et al., 2007; Murayama et al., 2007). Dbl family GEFs also shown to be regulated by gene expression, e.g. Ect2 (Scoumanne and Chen, 2006); subcellular localization, e.g. Net1 (Schmidt and Hall, 2002b) and by homodimerization, e.g. β-Pix (Kim et al., 2001).

1.3.5. GEFs as therapeutic targets

Spatio-temporal regulation of the Dbl proteins has been implicated to initiate activation of substrate Rho proteins and to control a broad spectrum of normal and pathological cellular functions (Schmidt and Hall, 2002a; Garcia-Mata and Burridge, 2007; Hall and Lalli, 2010; Mulinari and Hacker, 2010; Mulloy et al., 2010). Thus, it is evident that members of the Dbl protein family are attractive therapeutic targets for a variety of diseases (Bos et al., 2007; Loirand et al., 2008; Vigil et al., 2010). Inhibitors that target specific RhoGEFs have been discovered by high throughput screens. The first example was an aptamer screen, in which peptides coupled to thioredoxin were selected in yeast for their binding to the second DH domain (which specifically activates RhoA) of TRIO (Schmidt et al., 2002). This identified a potent inhibitor of TRIO, which was subsequently optimized to inhibit its oncogenic splice variant TGAT (Bouquier et al., 2009a). The corresponding optimized peptide was active in cells *in vitro* and reduced TGAT-induced tumour formation in xenograft models. Another assay screened a small chemical

compound library by monitoring the interaction of the Rho with an effector in the presence of a co-expressed GEF (Blangy et al., 2006). This yeast three-hybrid assay identified several inhibitors of RhoG activation by TRIO. One of these, ITx3, was specific and active in cell based assays (Bouquier et al., 2009b). Screening using a fluorescence polarization guanine nucleotide-binding assay also identified small molecule inhibitors of LARG stimulated RhoA nucleotide binding *in vitro* (Evelyn et al., 2009). Through computational screening of the surface of RAC1 that is known to interact with GEFs, the small molecule NSC23766 was discovered, which inhibited activation of RAC1 by the Rac-specific GEFs TRIO and TIAM1, but not GEF activation of RhoA or Cdc42 *in vitro* and in cells (Gao et al., 2004). Using a similar strategy, and using structural information from NSC23766 in complex with Rac1, five additional small molecules, that were structurally unrelated to NSC23766, were discovered that could specifically block Rac activation by GEFs (Ferri et al., 2009). These molecules do not directly target GEFs, and are likely to lack GEF specificity as they would block the surface of Rho protein and thus activation by various GEFs. They could nonetheless provide an interesting approach to block GEF activation of Rho proteins that are important in cancer.

1.4. GTPase activating proteins (GAPs)

The intrinsic GTPase activity of Ras superfamily proteins is accelerated by GTPase activating proteins (GAPs), which act to attenuate GTPase signaling by accelerating the conversion of bound GTP to bound GDP. For the small GNBPs like Ras, Rho, Ran, Rab and Arf, specific GAPs catalyze GTP hydrolysis. All GAP proteins which are different at the sequence level, utilize certain common mechanism and some divergent features to accelerate GTP hydrolysis of the cognate GNBP.

1.4.1. Rho GAP family proteins

Rho GTPase activating proteins (RhoGAPs) are the negative regulators of the Rho family. They function by accelerating the GTP hydrolyzing activity (GTPase reaction) of Rho proteins which is intrinsically long because of inefficient catalysis, by up to 5 orders of magnitude (Boguski and McCormick, 1993; Scheffzek et al., 1998; Bernards, 2003; Scheffzek and Ahmadian, 2005). Thereby, RhoGAPs inactivate Rho proteins by converting their active GTP-bound state to an inactive GDP-bound state and inorganic phosphate (P_i) (Fig. 4). The GTPase reaction is of great medical significance, since any disruption of this reaction, caused by inhibitory mutations in genes encoding for the GAP proteins, results in a persistent downstream signaling. The first realization that GTPases needs GAPs for their down regulation came from the finding that microinjection of recombinant GTP-bound Ras into living cells result in faster GTP hydrolysis than *in vitro* (Trahey and McCormick, 1987). This finding led to the purification of the first GAP for Ras GTPases, p120RasGAP, in 1988 (Trahey et al., 1988; Peck et al., 2002; Bernards, 2003). The first RhoGAP, p50RhoGAP, was identified by biochemical analysis of human spleen cell extracts with recombinant RhoA (Garrett et al., 1989) and later lead to the identification of other RhoGAP conating proteins: chimaerin and BCR whose amino acid sequence was related to p50 RhoGAP with GAP activity (Diekmann et al., 1991). Since then more than 80 RhoGAP containing proteins have been identified in eukaryotes, ranging from yeast to human (Lancaster et al., 1994; Moon and Zheng, 2003). The RhoGAP domain (also known as Bcr-homology, BH domain) containing proteins are present throughout the genome and rarely cluster in specific chromosomal regions (Peck et al., 2002). The RhoGAP family defines by the presence of conserved catalytic GAP domain which is sufficient for the interaction with Rho proteins, mediating accelerated catalysis (Scheffzek and Ahmadian, 2005). Beside their signature RhoGAP domain, most

of the RhoGAP family members are frequently accompanied by several other functional domains (Fig. 9) implicated in regulation, membrane targeting, localization and potential phosphorylation sites, indicating the complexity in the regulation of GTPase activity.

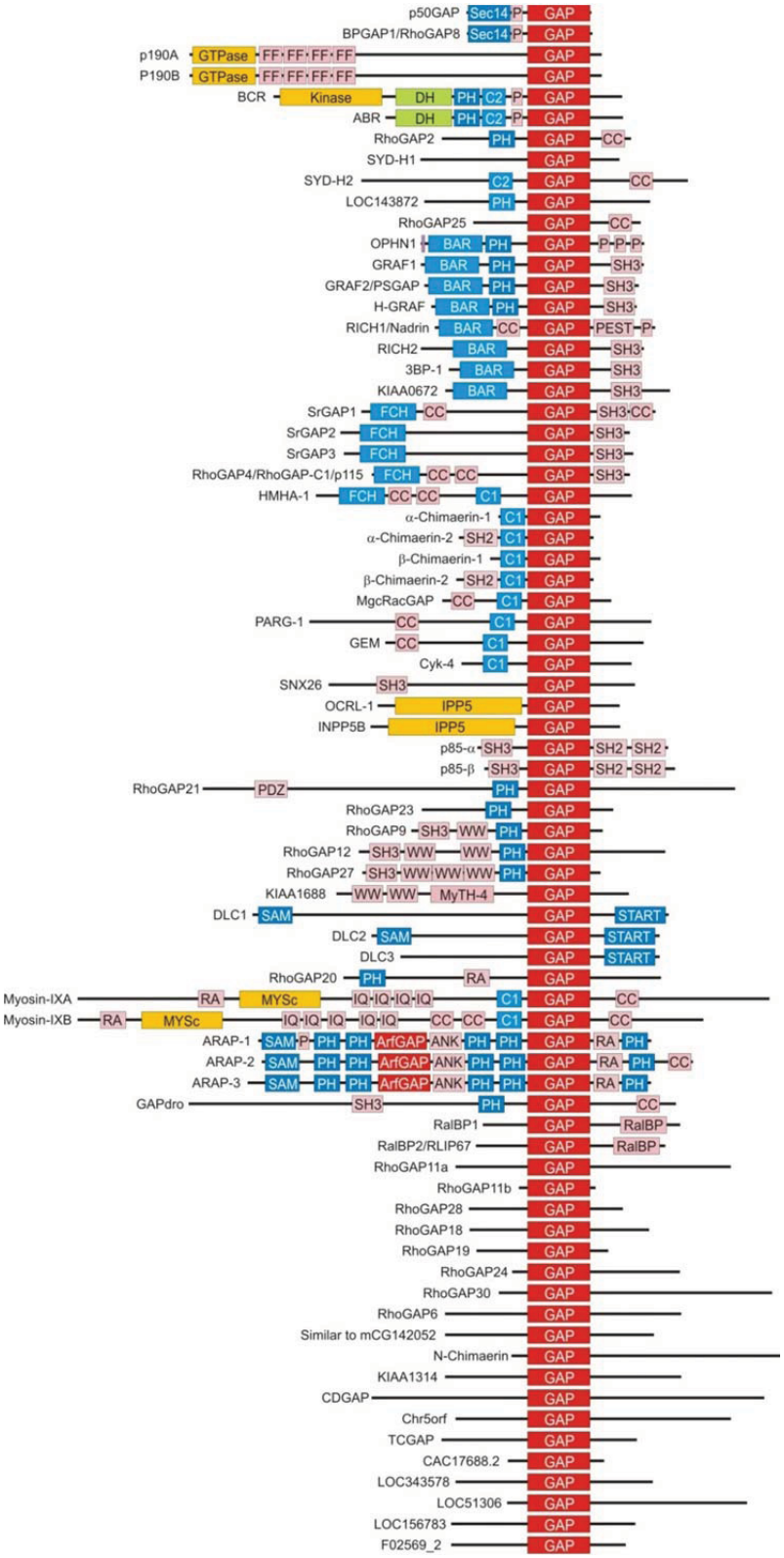

Figure 9. Domain organization of RhoGAPs
The domain organization of RhoGAPs are illustrated approximately to scale. The catalytic RhoGAP (GAP) domain is represented in red. Most of the RhoGAPs are multimodular proteins and have a number of functional domains that thought to mediate cross-talk between Rho proteins and other signaling pathways. Other functional domains are colored in blue, pink and orange on the basis of their properties to bind membrane, proteins and other functional domain, respectively. Abbreviations for domains are as follows: ANK, Ankrin Repeat; BAR, Bin/amphiphysin/Rvs; ArfGAP GTPase Region for Arf GTPases; C1, cysteine-rich phorbol ester binding; C2, calcium-dependent lipid binding; CC, predicted coiled-coil oligomerization region; DH, Dbl Homology, FCH, Fes/CIP4 homology; IQ, calmodulin-binding motif; IIP5, Inositol 5-phosphatase catalytic; Kinase, serine/threonine kinase; P, proline-rich SH3/WW target; PH, pleckstrin homology; RA, Ras-associating; Sec14, Homology to Yeast PI-transfer Protein Sec14p; START, StAR (steroidogenic acute regulatory)-related lipid transfer; SAM, sterile α motif, SH3, Src Homology 3; SH2, Src Homology 2; W, two signature Tryptophan (WW), proline-rich binding.

1.4.1.1. Structural and functional characteristic of RhoGAP domain

The GAP domain of RhoGAP family consists of approximately 150 amino acids and share high sequence similarity within the family. Although RhoGAP domain has no similarities at the amino acid level to other members of the RasGAP family, all RasGAP family members resembles with each other in their tertiary structure (Rittinger et al., 1998; Scheffzek et al., 1998). Comparative structural analysis of RhoGAP domain with other GAPs of Ras families suggest that GAP domains of Ras families are evolutionary related (Scheffzek et al., 1996; Rittinger et al., 1998) and the catalytic domain of RhoGAPs share a core structural fold. RhoGAP domain is made up of seven α-helices and highly conserved catalytic arginine (arginine finger) (Arg-85 p50RhoGAP numbering) residue which reside in a surface loop (Fig. 10). The functional characteristic of RhoGAP domain is a pair of conserved basic residues: catalytic arginine (arginine finger) and lysine (Arg-85 and Lys-122 in p50RhoGAP numbering, respectively) (Barrett et al., 1997).

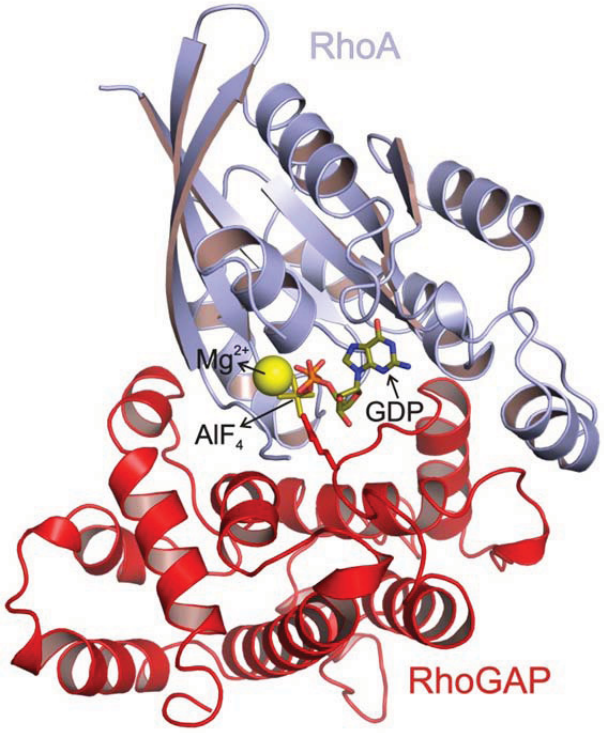

Figure 10. **Structure of RhoGAP**
Structure of transition state analogue of RhoA·GDP·AlF_4 (PDB accession code 1TX4). RhoGAP (red), RhoA (light blue), arginine finger (red, stick model), Mg2+ (olive, sphere), AlFx (olive), GDP (multicolor, stick model).

1.4.1.2. The mechanism of GAP domain mediated GTP hydrolysis

Crystallographic studies of RhoGAP domains in complex with Cdc42 bound to GppNHp, RhoA/Cdc42 bound to GDP·AlF_4 (Rittinger et al., 1997a; Rittinger et al., 1997b; Nassar et al., 1998) and RhoA bound to GDP·MgF_3 (Graham et al., 2002) gave insight into the catalytic mechanism of GTP hydrolysis stimulation. The GTPase reaction,

as part of the switch mechanism, leads to changes in the conformation of GTPases, especially in the flexible and mobile loops known as the switch regions (Bourne et al., 1990; Vetter and Wittinghofer, 2001; Dvorsky and Ahmadian, 2004). The RhoGAP interacts with the switch I and II regions as well as the P-loop of Rho protein. The GAP domain accelerates the intrinsic GTP hydrolysis of Rho proteins by two ways: (i) directly chemical contributing to the catalysis by the insertion of the catalytic arginine from GAP domain into the active site of RhoGTPase. This establishes contacts with main-chain carbonyl of Gly-12 (Rac1 numbering) and helps in stabilizing the GTP-hydrolysis transition state (Rittinger et al., 1997a), and (ii) stabilizing the charges formed during the transition state of GTP hydrolysis and positions the catalytic glutamine residue (Gln-61 Rac1 numbering- responsible for positioning of the water molecule for GTP-hydrolysis) of RhoGTPase to coordinate with nucleophilic water molecule (Rittinger et al., 1997b). RhoGAP also stabilize the switch regions of RhoGTPases by interacting to the residues of associated with intrinsic GTPases activity (Fidyk and Cerione, 2002). The inositol polyphosphate 5-phosphatase GAP domain, which lacks the conserved arginine, is devoid of activity (Jefferson and Majerus, 1995). The BH domain from the phosphoinositide3-kinase p85α-subunit has the critical arginine at approximately same position as p50rhoGAP, yet is inactive toward the GTPases so far tested (Musacchio et al., 1996). It was shown that p85α-subunit binds to Cdc42 and Rac1 but does not show GAP activity (Tolias et al., 1995).

1.4.2. Overabundance and diversity

Using database searches, 84 distinct RhoGAP domain containing proteins are found to encode in human genome whereas the number of Rho family proteins which need to be regulated by GAPs is 20 (excluding constitutively active Rho proteins Fig. 3). Thus number of RhoGAPs is nearly four-fold higher than that of their targets, the Rho proteins which means that multiple GAPs are capable of activating the same GTPase. It indicates the impressive and complex feature of these RhoGAP proteins and raises the question on the need of so many RhoGAPs to regulate RhoGTPases.

The overabundance of RhoGAPs implies that they must be tightly regulated in the cell so that all RhoGTPases are not always turned-off. This overabundance might be explained by: i) tissue-restricted expression, ii) specificity for only a single GTPase, and iii) regulation of specific Rho signaling pathways, respectively. In addition, some GAP

domains simply serve as a recognition module. Hence, the RhoGAPs act as effector or scaffold proteins, mediating cross-talk between Rho GTPases and other signaling pathways. The involvement in additional pathways is also assisted by their multi-domain feature (Fig. 9), what makes them not only signal terminators but pivotal players in many biological processes (Tcherkezian and Lamarche-Vane, 2007).

1.4.3. Specificity of RhoGAP domains

The occurrence of more than 70 RhoGAPs to regulate 22 Rho proteins in humans raise the how different RhoGAPs recognize different Rho GTPases. The substrate specificity of RhoGAPs for Rho proteins has been determined experimentally for fewer than half of the known RhoGAPs. Some RhoGAPs show specificity towards single Rho protein whereas some display a broader specificity i.e. active on more than one Rho proteins (Van Aelst and D'Souza-Schorey, 1997; Bernards, 2003). For example p190RhoAGAP shows GAP activity towards RhoA primarily (Ridley et al., 1993), p50RhoGAP shows GAP activity towards both Cdc42 (Barfod et al., 1993) and RhoA (Lancaster et al., 1994), RhoGAP6 specific for RhoA (Prakash et al., 2000), ARHGAP15 specific for Rac1 (Seoh et al., 2003) and OPHN1 exhibits strong GTPase-stimulating activity towards RhoA, Cdc42, and Rac1 (Eberth et al., 2009), however it should be emphasized that substrate specificity for most of the RhoGAPs has only been tested on three classical Rho prteins: RhoA, Rac1, and Cdc42, and most of the RhoGTPases have been not tested with any GAP. The GAP activities determined *in vitro* may be of limited value, in view of the spectrum of Rho family members, and the observation that their specificity can differ in vivo. Therefore, these results may have limited physiological relevance because it has been established that at least some of RhoGAPs, for example p190A (Settleman et al., 1992; Ridley et al., 1993), Myosin IXb (Wirth et al., 1996) have different GAP specificity *in vitro* and *in vivo* while Bcr shows activity on Rac1 but not on RhoA (Diekmann et al., 1991), β2 chimerin shows activity towards Rac1 but not for RhoA and Cdc42 (Caloca et al., 2003), 3BP1 shows activity towards Rac1 but not for RhoA and Cdc42 (Cicchetti et al., 1995), Rich1/Nadrin shows activity towards for Cdc42 and Rac1 but not for RhoA (Richnau and Aspenstrom, 2001) and RalBP1 has activity towards Cdc42 and Rac1 but not for RhoA (Cantor et al., 1995; Jullien-Flores et al., 1995). These all indicates that additional studies are also needed to determine the specificity of these RhoGAPs towards the other members of Rho family.

Therefore, we have focused on *in vitro* and *in silico* analysis to measure *in vitro* the activity of ten representative GAPs towards 15 members of the Rho family. This comprehensive analysis shows that in contrast to GEFs, RhoGAPs probably lack the specificity in cell free condition (unpublished). To explain this we have analyzed the interacting surface by comprehensively analyzing available complex structures of Rho and RhoGAP in PDB database and found that the Rho-GAP complex interface exhibits perfect complementarity and binding site of GAPs on Rho proteins is conserved as this is true for vice versa as well (unpublished) .

The scenario of the lack of specificity of RhoGAPs in cell free condition, can be different under cellular environment as it has been shown that the specificity of RhoGAPs is altered by lipids (Ligeti et al., 2004) or by phosphorylation (Minoshima et al., 2003). The study of Minoshima et al. showed that phosphorylation induced a shift in the specificity of MgcRacGAP (male germ cell Rac GAP) from Rac/Cdc42 towards RhoA (Minoshima et al., 2003).

1.4.4. Regulation and GAP proteins functions

Although the apparent redundancy of RhoGAPS could be explained by tissue specific distribution, the majority of the GAPs are widely expressed therefore it become important for the cell to regulate these important regulators, RhoGAPs, very tightly to prevent unwanted events as affect of persistent downregulated signaling . To ensure a stringent regulatory control the RhoGAPs are controlled at different levels indicating that region outside the RhoGAP domain (Fig. 9) must then determine the specificity of RhoGAPs. Numerous mechanisms have been shown to affect the catalytic activity, substrate specificity of RhoGAPs e.g. autoinhibition (GRAFGAP, OPHN1GAP) (Eberth et al., 2009), post-translational regulation: phosphorylation (p190GAP, MgcRacGAP) (Minoshima et al., 2003), and regulation by lipid-binding: PH or C2-domains (Ligeti et al., 2004), protein-protein interactions (DLC1/p120RasGAP) (Yang et al., 2009; Jaiswal et al., *in prep*.) and subcellular distribution as specific co-localization of RhoGAPs with Rho proteins at the membrane e.g. by a scaffolding protein (Bernards, 2003). In this thesis regulation of DLC1RhoGAP have been studied in detail (Scholz et al., 2011; Jaiswal et al., *in prep*.).

1.5. DLC family RhoGAPs

The human genome encodes more than 70 RhoGAPs that share a conserved GAP domain (Fig. 9). One subgroup of the human RhoGAPs contains DLC1 (also known as STarD12 or ARHGAP7), a human homolog of the rat p122RhoGAP (Yuan et al., 1998). By means of quantitative RT-PCR assay, it was determined that DLC1 is widely expressed in normal tissues, with high abundance in the lung and ovary, and moderately in the thyroid, spleen, intestine and kidney. The adrenal gland, liver and pancreas exhibit the lowest expression (Ko et al., 2010). There are two additional members of the DLC family are DLC2 (also known as StarD13 or ARHGAP37) and DLC3 (also known as STarD8 or ARHGAP38). DLC2 has a broad tissue distribution, with the highest levels in the brain, heart and liver (Ullmannova and Popescu, 2006; Durkin et al., 2007a). DLC3 is also detected in a broad spectrum of human tissues, with high abundance in the lung, kidney and placenta (Durkin et al., 2007a)(19). Together they constitute the DLC subfamily of RhoGAP proteins. The highest sequence conservation among these three proteins is found in their RhoGAP domain.

1.5.1. Deleted in liver cancer 1 (DLC1)

DLC1 was first identified in primary human hepatocellular carcinoma (HCC). Thus, it was proposed that DLC1 is a candidate tumor suppressor (Yuan et al., 1998). DLC1 now is endorse as a novel bona fide tumor suppressor gene because it is frequently deleted, inactivated or down-regulated in a variety of cancers and loss of its expression has subsequently been shown in various other tumor types, ranging from colon, breast to prostate (Durkin et al., 2007b). Deletion of the DLC1 in tumors is either caused by chromosomal deletions or suppression of its expression due to promoter methylation (Kim et al., 2003). Somatic mutations in the coding regions are rather uncommon, but have been recently reported (Durkin et al., 2007b; Liao and Lo, 2008). DLC2 and DLC3 the other members of DLC family have been linked to similar inhibitory effects on cell growth and function (Leung et al., 2005; Durkin et al., 2007a).

1.5.2. Domain organization of DLC family RhoGAPs

DLC family RhoGAPs are multidomain proteins and consist of three distinct domains: the sterile α motif (SAM) localized at its N-terminus, a conserved RhoGAP

domain found close to a central phosphorylation region and the steroidogenic acute regulatory(StAR)-related lipid transfer (START) domain at the C-terminus. SAM domain constitutes a very abundant protein-protein interaction motif. START domain is found in 15 mammalian proteins and predicted to interact with and/or transfer lipids by forming a hydrophobic tunnel. However, the proteins interacting with DLC1 START domain remain unknown and its function has to be further determined (Durkin et al., 2007b; Lukasik et al., 2011). The DLC1 encodes a 1091-amino acid protein with a predicted molecular mass of 122 kDa. The DLC2 encodes a 1113-amino acid protein with a molecular weight of 125 kDa, whereas the protein product of the DLC3 transcript has 1103 amino acids with a calculated molecular mass of 121 kDa.

The region between the SAM and RhoGAP domains displays little sequence similarity with known protein modules and large stretches of this middle region are predicted to adopt no globular conformation, conferring flexibility. This sequence is rich in serine residues and computer analysis revealed numerous potential phosphorylation sites (Scholz et al., 2009). In addition, several proline-rich segments within this region could possibly act as docking sites for proline recognition domains such as Src homology 3 (SH3) modules (Durkin et al., 2007b). Another important feature of this large unstructured sequence is a focal adhesion targeting (FAT) motif that mediates the association with the SH2 domain of tensins, a family of focal adhesion proteins (Liao et al., 2007).

1.5.3. RhoGAP activity of DLC family

In vitro studies revealed GAP activity of DLC1 for all three small GTPases of the Rho subfamily – RhoA, RhoB and RhoC and to a lesser extent for Cdc42 (Healy et al., 2008). Comparative study of all three members of DLC family for 12 Rho proteins revealed that DLCGAP can activate all 12 Rho proteins with highest activity towards RhoA (unpublished) . The data also indicate that the DLC family members are lousy RhoGAP and their catalytic efficiency is not in the order of other efficient RhoGAP (Jaiswal et al., *in prep*.). The study done for recently phosphorylation site within its GAP domain was identified as well which modulated its GAP activity (Scholz et al., 2011).

1.5.4. Biological function of DLC1 and its involvement in cancer

As a protein with GAP activity for the Rho family, a major function of DLC1 is certainly the regulation of cytoskeletal rearrangement and morphological changes. Thus, overexpression of DLC1 induced a rounded morphology and extensive membrane protrusions associated with the disassembly of actin stress fibers and disruption of focal adhesions, the processes which are known to be reversely regulated by Rho proteins (Sekimata et al., 1999; Kim et al., 2007). The impact of such activity is pivotal for the cell fate. Knockout mouse studies revealed an essential role of DLC1 for embryonic development. Homozygous mutant embryos died before 10.5 days post coitum with defects in the neural tube, brain, heart and placenta (Durkin et al., 2005).

Evidence for participation in tumor suppression firstly derived from experiments with ectopic expression of DLC1 in cancer cell lines lacking the endogenous protein. Restoration of DLC1 limited proliferation, colony formation and anchorage-independent growth in soft agar in hepatocellular, breast and lung cancer cell lines (Yuan et al., 2003; Yuan et al., 2004; Wong et al., 2005). Furthermore, inhibition of cell proliferation in HCC and renal cell carcinoma cells was associated with the induction of apoptosis (Zhou et al., 2004; Zhang et al., 2009). In nude mice the *in vivo* tumorigenicity was abolished (Yuan et al., 2003). Moreover, DLC1 was defined as a metastasis suppressor in breast cancer cells (Goodison et al., 2005). This role is consistent with re-expression studies, demonstrating the inhibition of migration and invasion in HCC, breast, lung and ovarian cancer cell lines (Goodison et al., 2005; Syed et al., 2005; Wong et al., 2005; Kim et al., 2007; Qian et al., 2007). The consequences of DLC1 ablation have only recently been investigated. siRNA mediated knockdown in breast cancer cell lines caused a dramatic increase in migration, which is in line with the opposite effects upon overexpression (Holeiter et al., 2008). With the use of a novel mouse model of liver cancer, Xue et al. provided evidence for the *in vivo* function as a bona fide tumor suppressor. They showed that shRNA-induced loss of DLC1 cooperated with c-Myc and p53 deficiency to promote the formation of liver tumors (Xue et al., 2008).

Many studies support the dependency of DLC1 tumor suppressive function on its GAP activity, because they reported that mutants lacking GAP activity were inactive with regard to cell growth inhibition (Wong et al., 2005). In addition, mutational analyis of cDNAs isolated from cancer patient samples detected two mutations within the linker region of DLC1 that resulted in a significant reduction of Rho GAP activity, impairing

the suppression of tumor cell growth (Liao and Lo, 2008). The mechanism of cell motility regulation by DLC1 was further examined and also shown to be GAP dependent. Inactivation of RhoA by DLC1 was demonstrated to take place preferentially at the leading edge of cellular protrusions, inhibiting the activation of the downstream effector Dia1, which in turn results in inhibition of directed cell migration (Healy et al., 2008; Holeiter et al., 2008). Nonetheless, evidence for GAP independent tumor suppressor activities has recently emerged. Thus, the introduction of a GAP-inactive mutant of DLC1 in lung cancer cell lines was also able to reduce colony formation, anchorage-independent growth in soft agar, cell migration and invasion (Healy et al., 2008). Further investigations will be needed to figure out the underlying mechanism and discover possible involved protein interaction partners and signaling pathways.

1.5.5. DLC1 regulation

The current knowledge about regulation of DLC1 is very limited. At the transcriptional level genetic and epigenetic mechanisms contribute to the control of its cellular concentrations. Thus, chromosomal deletion and hypermethylation of its promoter lead to loss of DLC1 expression (Yuan et al., 2003; Wong et al., 2005). Little is known about the regulation at the protein level. Screening for DLC1 protein interaction partners by a yeast-two-hybrid approach revealed tensins, a focal adhesion protein family of four related members (tensin 1/2/3 and cten) that interact with the cytoplasmic tails of β integrins, as the first binding partners of DLC1 (Yam et al., 2006; Liao et al., 2007; Qian et al., 2007). The association is mediated by the tensin Src homology 2 (SH2) domain and depends on tyrosine 442 in DLC1 but is phosphorylation independent. Through this interaction DLC1 is targeted to focal adhesions, which is essential for its biological activity (Qian et al., 2007). DLC1 was also reported to localize in caveolae (cholesterol-rich flask shape invaginations of the plasma membrane), where it interacts with caveolin-1, but the function remains unclear (Yam et al., 2006). By contrast, the interaction identified with p120RasGAP, a protein, which promotes the inactivation of Ras, displays a negative regulation of DLC1 function by inhibiting its GAP and growth-suppressing activities (Yang et al., 2009). From our i*n vitro* fluorescence spectroscopic analyses of purified recombinant proteins we have determined that: i) only the SH3 domain of p120RasGAP, specifically and selectively inhibits the RhoGAP activity of all three DLC isoforms, 2) unlike classical PxxP motif-recognizing SH3 domains, the

interaction mode of p120RasGAP SH3 domain is unique and utilizes different amino acids in order to bind and to displace the catalytic arginine finger of the GAP domain of DLC1 (Jaiswal et al., *in prep*.).

Posttranslational modifications such as phosphorylation are a common theme in protein regulation. DLC1 was also found to contain several phosphorylated sites by mass spectroscopic analysis (Scholz et al., 2009; Scholz et al., 2011). Two serines within the middle region (Ser-327 and Ser-431) and one serine in RhoGAP domain (S-807) were shown to be phosphorylated by protein kinase D (PKD), stimulating the association with 14-3-3 proteins. This interaction resulted in the inhibition of DLC1 GAP activity. In addition, DLC1 was found to undergo rapid nucleocytoplasmic shuttling but its nuclear function remains to be determined. Through binding to 14-3-3 adaptors DLC1 shuttling was blocked, retaining the protein cytosolic. Thus, 14-3-3 protein interaction reveals an additional mechanism that contributes to regulation of DLC1 activity and compartmentalization (Scholz et al., 2009).

A recent study provides evidence for a novel mechanism of DLC1 regulation. A polybasic region adjacent to the RhoGAP domain was identified to mediate phosphatidylinositol (4,5)-bisphosphate (PIP2) binding, stimulating DLC1 GAP activity and, thus, downregulating Rho signaling (Erlmann et al., 2009). Hence, also the influence of lipid interaction contributes to the regulation of DLC1 tumor suppressive functions.
Certainly, several additional mechanisms might be involved in activity control, subcellular distribution and protein turn-over. The intrinsic SAM domain, for example, was shown to act as autoinhibitory domain of the RhoGAP activity (Kim et al., 2008). However, it is not known how this inhibition takes place. From our study both isolated SAM and START domain have no effect on DLC1 RhoGAP activity however the full length DLC1 shows lower activity than isolated GAP domain alone (Jaiswal et al., *in prep*.). This implicate that DLC1 f.l. GAP activity is autoinhibited and the central phosphorylation region could have the impact on auto-inhibition (Jaiswal et al., *in prep*.).

2. CHAPTER 1

Biochemical assays to characterize Rho family proteins*

Background: To characterize RhoGTPase and their interactions with its regulators and effector proteins, not only structural information, but also details of kinetics and thermodynamics of the processes involved in are required. There are a number of methods that can be used to characterize the specificity, strength, and stoichiometry of such intermolecular interactions, to understand the effect of binding on the protein structure, and, ultimately, to obtain insights about their biological functions.

Results: This chapter describes (i) detailed protocols for the expression and purification of Rho GTPases, effector binding domains and catalytic domains of GEFs and GAPs, (ii) the preparation of nucleotide-free and fluorescent nucleotide-bound Rho GTPases and (iii) methods of monitoring of the intrinsic and GEF-catalyzed nucleotide exchange, the intrinsic and GAP-stimulated GTP-hydrolysis and the effector-interaction with active GTPase (three alternative approaches) using two different fluorescently labeled guanine nucleotides for the biochemical analysis of Rho GTPases.

Conclusion: Fluorescence spectroscopic methods, allows real-time monitoring of the interaction of RhoGTPases with GEFs, GAPs, and effectors under single turnover conditions at submicromolar concentrations, and quantification of the kinetic and equilibrium constants.

Significance: These methods are highly sensitive, quantitative, and are efficient to explain the complex mechanism utilized by Rho and their regulatory proteins GEFs, GAPs, and GDIs as well as effectors. They are used for analyzing most basic physical mechanism while the reaction is in process i.e. in real-time and also the influence of cofactors such as phospholipids on nucleotide exchange without interference from other cellular factors.

To characterize Rho proteins and to study their interaction with GEFs, GAPs, GDI and effectors for gaining insights into their biological functions two primary assays are in use: *in vitro* and *in vivo*. In the study entitled "Biochemical assays to characterize Rho

* ***Enclosure 1***

family proteins" (enclosure 1) we describe the methods of in vitro analysis for RhoGTPase characterization. *In vitro* analysis is powerful technique as it provides: (i) proper quantification and validation of reagents, (ii) titrations and (iii) time courses, which is difficult or impossible to perform in cell-based assays. Primarily investigated *in vitro* methods for characterization of Rho proteins are either solid phase methods like radioactive ligand overlay, pulldown assays or yeast two hybrid studies. These methods are often not sufficient to determine the specificity of regulation and to quantify the activity of recombinant proteins and many of the potential interactions defined by these methods require a more detailed analysis of their kinetics by appropriate real-time methods.

Fluorescence spectroscopy has provided vital insights in this challenging field and fluorescent guanine nucleotides are often ideally suited to fulfill these criteria, to obtain a detailed picture of the molecular switch function of Rho GTPases and their interaction with regulators and effectors. The most common guanine nucleotide analog carry either mant (N-methylantraniloyl) (Hiratsuka, 1983), BODIPY (McEwen et al., 2001), tamra (2'(3')-O-(N-ethylcarbamoyl-(5"-carboxytetramethylrhodamine) amide) (Eberth et al., 2005) and Cy3 (unpublished). This study (enclosure 1) (Jaiswal et al., 2012a) describes the application of two fluorophores: mant and tamra-labeled guanine nucleotide analogs to monitor in real time the biochemical properties of Rho GTPases. These fluorescent reporter groups are located on either the 2'or 3'oxygen of the ribose of GDP and GTP-analogues (Fig. 11).

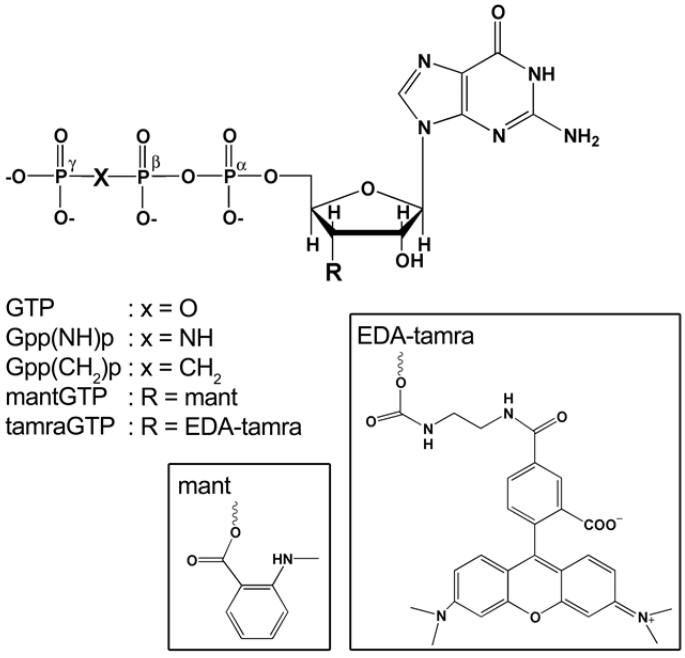

Figure 11. Chemical structure of the guanine nucleotide derivatives
The chemical structures of the guanosine nucleotide derivatives used and described in this chapter are shown. Unlabeled fluorescent nucleotides contain an OH-group at the position R. (Jaiswal et al., 2012a)

These fluorescent guanine nucleotides are suitable to characterize RhoGTPases as it is known that they do not grossly disturb the biochemical properties of the GTPase and the fluorescence reporter group is sensitive to changes in the local environment to produce a sufficiently large fluorescence change during GTP hydrolysis, nucleotide exchange of nucleotides as well the interaction of Rho proteins with their several regulatory proteins (GEFs, GAPs, GDIs, effectors) (Ahmadian et al., 2002; Hemsath and Ahmadian, 2005; Eberth and Ahmadian, 2009). Thereby enable to determine the binding affinities of nucleotides and effector domains as well as to evaluate the GEF-catalyzed nucleotide exchange and the GAP-stimulated GTP-hydrolysis activities, respectively. This chapter presented examples for quantitative characterization of (i) intrinsic and accelerated nucleotide exchange reaction of RhoA by the catalytic domains of PDZRhoGEF, (ii) intrinsic and stimulated GTP-hydrolysis reaction of Cdc42 by the catalytic domain of p50RhoGAP, and (iii) Cdc42-interaction with the GTPase binding domain of the Wiskott Aldrich syndrome protein (three alternative approaches).

Fluorescence spectroscopic methods described in this study provide tools for studying the intercommunication of a GTPase with nucleotides and binding partners. Compared to qualitative assays (e.g. the filter binding assay or thin layer chromatography, which contain between 3 and 6 data points), these fluorescence methods allow to monitor the activity of GEFs and GAPs in real time as well as the interaction with effectors at which every single measurement consists of at least 400 data points per reaction trace. These assays are highly sensitive and, in principle, reproducible provided that the proteins and reagents are carefully prepared from high purity materials and tested for quality.

3. CHAPTER 2

New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm*

Background: Intrinsic properties of most of the Rho proteins are uncharacterized. Based on three most extensively studied members RhoA, Rac1 and Cdc42, Rho proteins are believed to persist in resting cells as inactive GDP-bound form alone or alternatively in complex with GDIs.

Results: Comprehensive investigation 15 members of the Rho family regarding their intrinsic properties revealed the critical differences not only among the highly related Rho and Rac isoforms but also among all members of Rho family. RhoD and Rif surprisingly exhibit faster nucleotide exchange than GTP hydrolysis.

Conclusion: Faster nucleotide exchange than GTP hydrolysis exhibited by RhoD and Rif shift the equilibrium largely towards GTP-bound state under resting conditions. Therefore, RhoD and Rif have ruled over the conventional switch mechanism of Rho family proteins, and shifting the paradigm of classical regulation of small GTP binding proteins.

Significance: This study provoked the verification of conventional regulation of Rho switch mechanism by conducting more detailed biochemical analysis of Rho family proteins in the interplay with 3 GDIs, 74 GEFs and 84 GAPs, and their interaction with and activation of more than 100 effector proteins.

The study of the intrinsic properties of Rho family proteins (nucleotide binding, nucleotide dissociation and GTP hydrolysis) was performed by several groups (Zhang et al., 2000; Fiegen et al., 2004; Jaiswal et al., 2011). Despite of different experimental method and condition used in these studies, their valuable experimental data give us rough estimation on intrinsic biochemical behavior of Rho family proteins that: (1) affinity of Rho proteins to nucleotide binding is high and comparable to Ras and Rab

* ***Enclosure 2***

family, and (2) intrinsic nucleotide exchange rate and intrinsic GTP hydrolysis are extremely slow. Since intrinsic GTP hydrolysis of Rho proteins is faster than intrinsic GDP/GTP nucleotide exchange, Rho proteins in resting cells are believed to persist as inactive GDP bound form or alternatively in complex with GDIs (Dovas and Couchman, 2005). This scenario is based on the study of three classical Rho proteins: RhoA, Rac1 and Cdc42. Since individual Rho family proteins have sequence divergence, it is not appropriate to generalize the intrinsic property of individual Rho family proteins and also in context of cell each Rho proteins behave different in their local functional niche. Therefore, in order to compare the intrinsic properties all Rho family proteins one platform was set in the study entitled "New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm" by utilizing same method (fluorescence spectroscopic and HPLC method), experimental condition and highly purified recombinant 15 Rho proteins (Jaiswal et al., 2012c). This comprehensive investigation enabled us to analyze and compare sequence-structure-function relationships of fifteen members of the Rho family, including RhoA, RhoB, RhoC, Rnd1, Rnd2, Rnd3, Rac1, Rac2, Rac3, RhoG, Cdc42, TC10, TCL, RhoD and Rif, regarding their intrinsic biochemical properties: (i) nucleotide binding, (ii) nucleotide dissociation (k_{diss}), and (iii) GTP hydrolysis (k_{cat}).

2.1. High nucleotide binding affinity of Rho proteins

First association kinetics of GDP nucleotide to 12 Rho family proteins (Rnd proteins were excluded) was measured and this comparative analysis revealed that RhoA, Rac1, Rac3 and Cdc42 have strikingly rapid nucleotide association compared especially to that of RhoB, Rac2 and TC10, which are more than 100-fold slower. To complement the nucleotide binding properties, the GDP dissociation of Rho proteins were also measured and revealed that mantGDP dissociation from Rho proteins is very slow and lasts up to 36 h to be completed and this result to an extremely low dissociation rate constants (k_{diss}) for Rho proteins. Taken together, dissociation constants (K_d) calculated as k_{diss}/k_{ass} ratio revealed that the affinities of RhoA, Rac1 and Cdc42 proteins for mantGDP are in the high picomolar range, which were 1000-fold lower in the case of RhoD and Rif. The data obtained from this study revealed that the nucleotide binding affinity for most of the Rho family proteins is high and are in the same range as other small GTPase families such as Ras, Ran, Rab and Arf.

2.2. RhoD and Rif are unique in nature

RhoD and Rif proteins show relatively faster dissociation of mantGDP than other analyzed Rho proteins by almost three orders of magnitude. Interestingly, the nucleotide binding properties of RhoD and Rif are rather comparable to that of Rac1b, an alternative splice variant of Rac1 (Fiegen et al., 2004; Haeusler et al., 2006), having faster nucleotide dissociation as well.

While Rho proteins exhibit marked differences in their guanine nucleotide binding properties, the rates of GTP hydrolysis appeared to be rather similar. Interestingly, there are up to 5-fold differences in GTP hydrolysis rate constant ($_{kcat}$) for RhoD and Rif, therefore they belong to moderately hydrolyzing members of the Rho family.

2.3. Shifted paradigm of for RhoD and Rif

The rate constant for GTP hydrolysis (k_{cat}) for Rho proteins is conventionally higher relative to rate constant of nucleotide dissociation (k_{diss}). Therefore, majority of the Rho family proteins under resting conditions exist predominantly in the inactive, GDP-bound state (Fig. 12). From this study, a comparison of the rate constants for the GDP dissociation (k_{diss}) and the GTP hydrolysis (k_{cat}) clearly indicates that RhoD and Rif exhibit strikingly faster nucleotide exchange than GTP hydrolysis ($k_{diss} > k_{cat}$), which shifts the equilibrium of RhoD and Rif largely towards GTP-bound state in resting state (Fig. 12). This result is another milestone in canonical regulation of Rho GTPase cycle as RhoD and Rif have ruled over the conventional switch mechanism of Rho proteins. This study proposed that RhoD and Rif persist unlike the conventional members of the Rho family in the active state under resting (unstimulated) conditions.

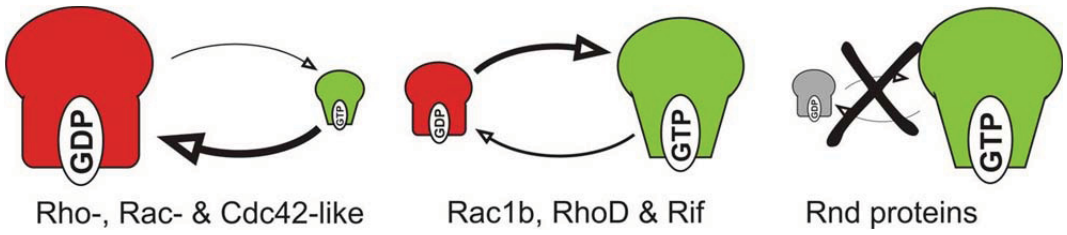

Figure 12. Differential intrinsic function and mechanism of Rho proteins
Conformational changes driven by an extremely slow nucleotide exchange and a relatively fast GTP hydrolysis is an attribute of canonical molecular switches keeping them in their GDP-bound, inactive state under resting conditions. The thickness of the arrows represents different magnitudes of the nucleotide exchange and hydrolysis reaction rates. (Jaiswal et al., 2012c)

4. CHAPTER 3

Mechanistic insights into specificity, activity and regulatory elements of the RGS-containing Rho-specific Guanine Nucleotide Exchange Factors p115, PDZ-RhoGEF (PRG) and Leukemia- associated RhoGEF (LARG)*

Background: In spite of intensive research there is little comparative analysis is available for individual regulator of G-protein signaling (RGS) domain containing Dbl GEFs LARG, p115, and PRG regarding their specificity, activity and regulation.

Results: LARG, PRG, p115 and p190 GEFs are specific for the Rho isoforms (RhoA, RhoB and RhoC). DH domain of LARG exhibits highest catalytic nucleotide exchange activity reported till now. The tandem PH domains of p115 and PRG efficiently contribute to the DH-mediated nucleotide exchange reaction but not in the association reaction. In contrast to the isolated DH or DH-PH domains, a p115 fragment encompassing both RGS and the DH domains, revealed a significantly reduced GEF activity supporting the proposed models of an intramolecular autoinhibitory mechanism for p115-like Dbl GEFs.

Conclusion: DH-PH domain determines the specificity. High catalytic nucleotide exchange activity of LARG is attributed to its faster association with RhoA. The N-terminus of the DH domain plays a crucial role in determining catalytic efficiency.

Significance: This study provides insight into specific structural features that contribute to large differences in the catalytic activity and binding kinetics between GEFs and Rho.

The common structural module of Dbl-family GEFs, which is responsible for stimulating the nucleotide exchange activity of Rho proteins, consists of a DH domain and an adjacent PH domain (Hoffman and Cerione, 2002; Rossman et al., 2005). In contrast to the conserved of the DH-PH tandem, the GEFs of the Dbl family also exhibit a variety of functional domain compositions and domain organizations (Fig. 5) (Zheng,

* ***Enclosure 3***

2001; Schmidt and Hall, 2002a; Rossman et al., 2005; Aittaleb et al., 2010), which link their GEF activity to specific signaling events. Interesting examples in this regard are regulator of G-protein signaling (RGS) domain-containing RhoGEFs such as p115, PRG and LARG (Fig. 5). The RGS domain at the N-terminus of p115 directly links the heterotrimeric G proteins $G\alpha_{12/13}$ to RhoA regulation and acts as a GTPase activating protein (GAP) for $G\alpha_{12/13}$ (Kozasa et al., 1998; Sternweis et al., 2007). The association of p115 with $G\alpha_{12}$ and $G\alpha_{13}$ has been suggested to activate its GEF function towards Rho proteins (Hart et al., 1996; Hart et al., 1998). A similar regulatory model has been proposed for PRG and LARG (Fukuhara et al., 1999; Fukuhara et al., 2000; Jackson et al., 2001; Booden et al., 2002; Swiercz et al., 2002; Vogt et al., 2003; Vazquez-Prado et al., 2004). Recently, Zheng *et al*. provided a direct biochemical evidence for an autoinhibitory RGS-mediated regulation of the DH domain (Zheng et al., 2009).

In spite of intensive research, there is little comparative analysis of these RGS-containing GEFs available. Thus, in this study (enclosure 3) (Jaiswal et al., 2011) we purified different protein domains of p190, p115, PRG and LARG and characterized them functionally regarding their specificity, activity and regulation with respect to each other. We have employed fluorescence spectroscopic methods utilizing both, GTPases loaded with fluorescently labeled guanine nucleotides (Hemsath and Ahmadian, 2005) and a fluorescent RhoA itself ($RhoA^{V33C}$-AEDANS abbreviated as fRhoA, prepared in this study) to determine quantitatively: (i) the specificity of four GEFs, p115, p190, PRG and LARG on six RhoGTPases, (ii) their catalytic constants (k_{cat}, K_m) towards RhoA, (iii) the association of RhoGEF with GDP-bound fluorescent RhoA (fRhoA) and (iv) the influence of other domains, such as the PH and RGS domain, and an N-terminal segment on the DH capability in both binding fRhoA·GDP and catalyzing mantGDP dissociation from RhoA. p190, a Rho-specific GEF (van Horck et al., 2001), was used as a control.

This comparative study shows that: (i) p115, p190, PRG and LARG GEFs specific for the Rho isoforms (RhoA, RhoB, RhoC) and inactive towards other members of the Rho family including Rac1, Cdc42 and TC10. (ii) Beside their specificity towards Rho isoforms it also show that all three (p115, p190 and PRG) were less efficient in the acceleration of nucleotide dissociation compared to LARG. (iii) RhoGEF-catalyzed exchange reaction is independent of the type of incoming nucleotide. (iv) The DH domain is highly efficient catalytic machine and the DH domain of LARG exhibits the highest catalytic activity reported for a Dbl protein till now, with a maximal acceleration of the

nucleotide exchange by 10^7-fold, which is at least as efficiently as reported for GEFs specific for Ran or the bacterial toxin SopE. (v) PH domain assists the nucleotide exchange reaction of p115 and PRG but not of p190 and LARG. (vi) Development of fluorescent RhoA (fRhoA) provided an attractive technique that enables to better understand both, the differential RhoA-binding characteristics of the RhoGEFs and the role the PH domains in the exchange reactions. (vii) An efficient catalytic activity of a GEF is dependent at least on two successive reactions: association with RhoA·GDP and exchange of the bound GDP for GTP proceeding via a high affinity nucleotide-free GEF·RhoA reaction intermediate. (viii) The tandem PH domains of p115 and PRG efficiently contribute to the DH-mediated nucleotide exchange reaction but not in the association reaction. (ix) A novel regulatory region at the N-terminus of the DH domain is involved in its association with GDP-bound RhoA monitored by a fluorescently labeled RhoA. (x) The N-terminal RGS-Linker of p115 controls negatively the DH activity and supports the proposed models of an intramolecular autoinhibitory mechanism for p115-like DblGEFs.

4.1.Structure-based interaction sequence matrix

Creation of "Structure-based interaction sequence matrix" is the gold of this study which enables us to explain how the specificity of the p15, p190, PRG and LARG RhoGEFs for the Rho isoforms is achieved. To create the matrix we first identified the contacting residues of the RhoA/PRG and RhoA/LARG complexes using the respective crystal structures (Derewenda et al., 2004; Kristelly et al., 2004) and aligned them to various DH-PH tandems and to the G domains analyzed in this.

This structure-based interaction sequence matrix reveals the specificity-determining residues from both the Rho-specific GEFs and the Rho isoforms which, strikingly linked together via ionic and H bonds.

4.2. Attributes of catalytic efficiency of the RhoGEFs

A striking finding of this study is that PRG and LARG exhibit higher GEF activity, which is two orders of magnitude higher as compared to p115 and p190. This is particularly interesting, because p115 belongs to the same subfamily of RGS-containing Dbl proteins as PRG and LARG (Fukuhara et al., 2001; Aittaleb et al., 2010). To explain the catalytic efficiency of PRG and LARG versus p115 we focused on the available

structural data. From our structure-based interaction sequence matrix, in which we inspected crystal structures of PRG (Derewenda et al., 2004) and LARG (Kristelly et al., 2004) in complex with RhoA, we selected nine variable residues that are in direct contact with the nucleotide-free form of RhoA and performed mutational analysis.

An explanation for the much lower efficiency of p115 GEF as compared to PRG and LARG resided from differences of the GEF association with the GDP-bound RhoA, which we obtained by developing a new method. This was convincing for an efficient catalytic activity of LARG, as activity of a GEF is dependent at least on two successive reactions: (i) association with RhoA·GDP and (ii) exchange of the bound GDP for GTP proceeding via a high affinity nucleotide-free GEF·RhoA reaction intermediate (Fig. 8).

4.3. Differential roles of the tandem PH domain

PH domains are best known for their ability to bind phosphoinositides with high affinity and specificity, although it is now clear that less than 10% of all PH domains share this property (Lemmon, 2007). Working with the Dbl family exchange factors consistently raises the question about the functional role of the tandem PH domain. Such an arrangement has been proposed to imply a crucial and unique functional interrelationship (Whitehead et al., 1997; Zheng, 2001). Our kinetic data of the exchange reaction imply that the PH domains contribute to the nucleotide exchange reaction mediated by the DH domain to different extend depending on the particular GEF. Compared to the activity of the isolated DH domain, the DH-PH tandem of PRG and p115 exhibited up to 5-fold enhanced exchange activities, respectively (Jaiswal et al., 2011). This finding is supported by several previous studies and indicates that the DH domains of p190 and LARG represent the entire catalytic machinery to accomplish Rho activation and that their PH domains do not contribute to the activity of Rho activation.

In summary, our data strongly support the conclusion that the DH domain of the RhoGEFs itself determines the specificity for binding RhoGTPases and represents very efficient catalytic machinery for the nucleotide exchange in a cell-free and membrane-free system. In cells, however, a set of additional domains and interactions are required for the shuttling, localization and activation of the GEFs (Robbe et al., 2003; Rossman et al., 2005; Garcia-Mata and Burridge, 2007).

5. CHAPTER 4

Deciphering the molecular and functional basis of Dbl family proteins: a meta-analysis*

Background: Most of the members of Rho and Dbl family are largely uncharacterized and this makes the analysis of specific upstream pathways complex.

Results: Not all Rho proteins, including RhoD and Rif, need GEFs. Dbl family proteins are divided in mono-, isoform- and oligo-specificity groups.

Conclusion: Catalytic efficiency of Dbl proteins is proportional to their association reaction.

Significance: Dbl family classification into distinct subfamilies opens doors to further cell-based research.

The existence of 74 Dbl proteins in human (Fig. 5) to regulate nucleotide exchange of 15 susceptible Rho proteins (Fig. 3) strongly suggests that a single Rho protein can be activated by several Dbl proteins to potentially regulate multiple signaling pathways. A literature survey showed that the current state of knowledge is limited to the activity of 44 Dbl proteins and to only Cdc42, Rac1, RhoA and in some cases also for RhoG using various methods and conditions. In spite of their values, data reported so far do not allow general conclusions about selectivity, efficiency and specificity, and this is due to a large variation of methods and experimental designs. To revise this status of knowledge we performed in this study (enclosure 4) (Jaiswal et al., 2012b) a meta-analysis by compiling our own data obtained from this study and previous published data regarding both biochemical data of describing GEF activity of the Dbl family proteins for their substrate Rho proteins and three-dimensional structures of Rho and Dbl proteins, and their complexes. This led us to evaluate effectively a sequence, structural and functional relationship of large set of Dbl (21) and Rho (12) proteins under cell-free conditions and to classify proteins of Dbl family proteins into distinct subfamilies regarding their substrate selectivity and signaling specificity.

* ***Enclosure 4***

5.1. Specificity of Dbl family proteins

Dbl family proteins can differentially couple to one or more of the 15 susceptible Rho family members dependent on their specificity to the substrate. The data obtained from kinetic analysis of 21 individual Dbl GEFs mediated nucleotide exchange for 12 Rho proteins show that the investigated Dbl proteins exhibit high selectivity for the Rho, Cdc42 and Rac proteins and show varying degree of specificity. On this basis Dbl proteins are divided in this study into three subgroups mono-, isoform-, and oligo-specific. (i) TrioN, ITSN1, ASEF and Vav2, and perhaps also hPem2 and Tuba are `mono-specific´ meaning that they exhibited by far the highest activity for one member of the Rho family; (ii) LARG, PRG, p115, p190 and Tiam1 are `isoform-specific´; (iii) Dbl, Dbs and PRex1 are `oligo-specific´ meaning that they are able to significantly accelerate the nucleotide exchange of 5 to 9 different Rho proteins.

5.2. Efficiency of Dbl family proteins

Another main finding of this study is the striking degree of differences and broad spectrum of catalytic efficiencies of 14 Dbl proteins for 12 Rho proteins, which range from 5-fold to almost 60,000-fold acceleration of the intrinsic nucleotide exchange. To illustrate this explicitly, we plotted all 168 pairs of Dbl and Rho proteins (y-axis) against fold activation (x-axis) in a numeric order starting with LARG-RhoA with the highest efficiency (57,000-fold) and ending with LARG-RhoD with no activity. Overall, the Dbl-Rho protein pairs were subdivided in five groups based on their catalytic efficiency to distinctly enhance the intrinsic nucleotide exchange of the Rho proteins: (i) high efficiency, (ii) intermediate efficiency, (iii) low efficiency, (iv) inefficient, and (v) inactive pairs.

There are two major mechanisms that may control the catalytic efficiency of the Dbl proteins under the conditions used in this study, either the association of the Dbl protein with the GDP-bound Rho protein or the nucleotide exchange reaction itself or both. To examine whether an association-controlled mechanism is a reason for the extreme differences in the catalytic efficiency, fluorescently labeled RhoA that allows real-time measuring of its association with Dbl proteins was used (Jaiswal et al., 2011). Four Dbl proteins (LARG, p190, Vav2 and TrioN) with differential efficiency towards RhoA were selected and their association was measured with inactive GDP-bound form

of RhoA. The data obtained from this analysis showed a clear correlation between both the nucleotide exchange and the association reactions. These data strongly suggest that the catalytic efficiency of the Dbl proteins is directly proportional to their association rate constant (k_{on}) of the GDP-bound Rho proteins.

5.3. Hotspots identification on protein interfaces

To address the question how the selectivity of the Dbl proteins for their substrate Rho proteins is achieved, two strategies were employed in this study to investigate systematically the sequence-structure-property relationship of the interaction between Dbl and Rho proteins. (i) The pairs of interacting residues were combined with two multiple sequence alignments of the Dbl and Rho proteins analyzed in this study to build up a structure-based interaction matrix. The corresponding matrix provided a complete overview of the conservation of respective amino acids utilized by the DH-PH and the Rho proteins. (ii) "structure-based conservation maps" of 12 Rho and 74 Dbl proteins was generated and projected on the complex structure of G domain of RhoA and DH-PH domains of LARG, respectively. The results of these analyses remarkably provided several novel insights into structure-function associations and evidences for the assignment of the Dbl family to subfamilies.

5.4. RhoD and Rif may not need GEFs

An interesting finding of this study is that none of the 21 Dbl GEFs investigated showed activity towards RhoD and Rif. This observation is rather interesting and emphasis towards the unique status of these two Rho proteins. We have shown recently that the GDP dissociation from RhoD and Rif, similarly to Rac1b (Fiegen et al., 2004) and Wrch1 (Shutes et al., 2006), is faster than their activity to hydrolyze GTP (Jaiswal et al., 2012c) and proposed that RhoD and Rif, unlike the conventional members of the Rho family, persist mainly in the active state under resting conditions (Jaiswal et al., 2012c). These combined observations indicate that RhoD and Rif proteins do not need to be regulated by GEFs if they are integral elements in slow cellular processes. However, RhoD and Rif are dependent of acute activation by GEFs in the course of fast signaling processes, such as regulation of actin dynamics (Gad and Aspenstrom, 2010). Results of this study strongly support the notion that members of the Dbl proteins family may not

play a role in an activation of RhoD and Rif or in other words we can state that atypical Rho proteins especially RhoD and Rif escape from canonical regulation by GEFs. And this highlights the existence of additional levels of regulation for atypical members of Rho family, for example, by other regulatory proteins like GAPs, and GDIs or by transcription, or protein degradation via ubiquitination (Visvikis et al., 2010; Doye et al., 2012). Further studies are required to understand the mechanisms nature of RhoD and Rif regulation.

5.5. Not all Dbl proteins are GEFs

From the 21 investigated Dbl family proteins in this study, seven Dbl proteins did not show any GEF activity towards any of the 12 investigated Rho proteins under experimental conditions. These include DH-PH domains of FGD4, FGD6, Abr, Bcr, Sos1, α-Pix, and β-Pix. To further investigate the reason of these protein not showing any GEF activity we looked first on their sequences. Multiple sequence analysis was performed separately for each Rho, Cdc42 and Rac specific GEFs. The conservation profile of the inactive GEFs with other active GEFs shows that these Dbl proteins contian most of the conserved interacting residues. Therefore, these inactive GEFs are addressed further as “GEF-like proteins” (unpublished). The existence of these GEF-like proteins further raises two questions: (i) why GEF-like proteins do not show any GEF activity, (ii) what is the role of GEF-like proteins in cellular context?

6. CHAPTER 5

The tumor suppressor protein DLC1 is regulated by PKD-mediated GAP domain phosphorylation*

Background: DLC1 is a tumor suppressor protein that is frequently down regulated due to deletion or promoter methylation in various tumor types. DLC1 contains a RhoGAP domain that appears to be required for its tumor suppressive functions. PKD-mediated phosphorylation of DLC1 on serines 327 and 431 initiates the binding of 14-3-3 protein and may thereby modulates its GAP activity.

Results: Novel PKD phosphorylation site serine 807 within the RhoGAP domain of DLC1 was identified. This phosphorylation has no impact on GAP activity of DLC1 in vitro. However, a serine to alanine mutation at 807 inhibits colony formation more potently than the wild type protein.

Conclusion: PKD-mediated phosphorylation of DLC1 negatively regulates DLC1 cellular function.

Significance: PKD-mediated phosphorylation and the role of DLC1 as tumor suppressor, gives the potential relevance of this phosphorylation to tumorigenesis.

The DLC1 gene was first isolated as a candidate tumor suppressor gene in primary human hepatocellular carcinoma and loss of expression has subsequently been shown in various other tumor types, ranging from colon, breast to prostate (Durkin et al., 2007). DLC1 is a GTPase activating protein (GAP) that exhibits in vitro GAP activity for RhoA, RhoB and RhoC, and to a lesser extent Cdc42 (Wong 2003; Healy et al., 2007).

DLC1 is frequently downregulated due to deletion or promoter hypermethylation. Those tumors that still express DLC1 may have developed other means to inactivate its regulatory function within the cell. For example, by DNA sequencing of prostate and colon cancer samples, two somatic mutations (T301K and S308I) in DLC1 were identified that impair RhoGAP activity and consequently the ability to inhibit cancer cell proliferation (Liao and Lo, 2008). Phosphorylation of the rat DLC1 protein in response to

* ***Enclosure 5***

insulin has been reported, however, the functional consequences of this phosphorylation are unknown (Hers et al., 2006). Previously PKD has been identified to phosphorylate DLC1 on serines 327 and 431, thereby creating recognition motifs for the phosphoserine-/phosphothreonine-binding 14-3-3 adaptor protein family (Scholz et al., 2009). 14-3-3 binding inhibited cellular DLC1 GAP activity, most likely by sequestration of DLC1 in the cytoplasm. Accordingly, DLC1-mediated inhibition of cell spreading was alleviated upon co-expression of 14-3-3 proteins and a DLC1 mutant defective in 14-3-3 binding (S327/431A) was more potent at inhibiting cell proliferation than the wild type protein (Scholz et al., 2009).

In this study (enclosure 5) (Scholz et al., 2011) the novel phosphorylation site within DLC1 on serine 807 was identified by mass spectrometry analysis. This phosphorylation site was of particular interest as it is located within the GAP domain of DLC1, raising the possibility that phosphorylation at serine 807 may be directly involved in the regulation of GAP activity. Later, from the analysis it was confirmed that this site matches the consensus motif of PKD (L/I/VxRxxS/T), a kinase that have been previously shown to phosphorylate DLC1 on serines 327 and 431 (Scholz et al., 2009). DLC1 phosphorylation via PKD on S807 site was confirmed by a phospho-S807-specific antibody and identified that the PKD family (PKD1, PKD2 and PKD3) of serine/threonine kinases are upstream kinases of DLC1 at serine 807.

Since serine 807 lies within the DLC1 GAP domain, it prompted to test whether *in vitro* GAP activity was altered by phosphorylation at this site. To this end, DLC1 wild type and the 807 phosphorylation site mutants were subjected to *in vitro* GAP assays with recombinant RhoA loaded with radiolabeled GTP. In this assay, GTP hydrolysis rates were similar in all cases and also measurements with Cdc42 as a substrate using fluorescence spectroscopy method did not reveal any differences, indicating that at least *in vitro* serine807 phosphorylation does not appear to impact on GAP activity of DLC1.

To rule over the possibility whether phosphorylation at S807 has affect on DLC1 activity in a cellular context, several assays were performed, which revealed that mutating S807 phosphorylation site to non-phosphorlatable alanine increases DLC1- mediated cell migration, while mutation to phosphorylation mimicking aspartate residue has the opposite effect. This study also demonstrated that S807A mutant increases the inhibition of colony formation in cancer cells transfected with DLC1.

Although this study did not find any significant changes in GAP activity towards RhoA *in vitro*, the DLC1 phosphorylation site mutants possessed distinct activities in

cellular assays. Though *in vitro* experiments were done with recombinant RhoA and Cdc42, it cannot be ruled out that phosphorylation specifically modulates substrate specificity towards another GTPase. For example, the specificity of MgcRacGAP switches from Rac and Cdc42 to Rho in response to phosphorylation by Aurora B kinase (Minoshima et al., 2003). Alternatively, phosphorylation can impact on protein localization and/or protein-protein interactions. DLC1 recruitment to focal adhesions is thought to be a prerequisite for biological activity, but the subcellular localization of the DLC1 S807 phosphorylation site mutants was undistinguishable from that of the wild type protein. Recently, the DLC1 GAP domain was reported to be inhibited by interaction with the p120RasGAP protein (Yang et al., 2009; Jaiswal et al., *in prep*.). The pulldown experiments, performed in this study did not observe any differences in p120RasGAP association with the DLC1 807 phosphorylation site mutants (data not shown), but it remains possible that complex formation with other still to be identified protein partners is affected by DLC1 phosphorylation on this site. Elucidation of the precise biochemical mechanism by which DLC1 phosphorylation on serine 807 modulates its activity may thus require screening for interaction partners at the proteomic level.

PKD is a family of serine/threonine kinases for which only a few physiological substrates have been identified thus far. The function of PKD has been studied best at the Golgi complex where it is known to regulate vesicular traffic to the plasma membrane (Wang, 2006). More recent studies suggest that PKD has additional functions associated with the regulation of cell shape, movement and invasion (Eiseler et al., 2009; Peterburs et al., 2009; Eiseler et al., 2010). The identification of the RhoGAP and tumor suppressor protein DLC1 as a PKD substrate thus creates a novel link between PKD, Rho signaling and cell transformation. The fact that PKD is activated downstream of Rho under certain conditions, which in turn would result in DLC1 inhibition by PKD-mediated phosphorylation implicates a feedback mechanism allowing amplification of Rho signaling. Previous report shows that PKD inhibits DLC1 cellular function through phosphorylation of serines 327 and 431 (Scholz et al., 2009). This study provides evidence that PKD additionally phosphorylates serine 807 contained within the DLC1 GAP domain. The fact that PKD negatively controls DLC1 through phosphorylation on multiple sites underscores the necessity for tight control of DLC1 cellular function to ensure correct Rho signaling.

7. CHAPTER 6

Identification of structural and functional determinants of the trans-inhibition of DLC tumor suppressor proteins by p120RasGAP*

Background: DLC1 RhoGAP interact with the SH3 (Src homology 3) domain of p120RasGAP.

Results: The SH3 domain of p120RasGAP, specifically and selectively inhibits the RhoGAP activity of DLC family RhoGAPs.

Conclusion: The interaction mode of SH3 domain of p120RasGAP is unique, unlike to classical PxxP motif-recognition.

Significance: The specific and selective interaction of DLC1 RhoGAP to p120RasGAP via SH3 domain highlights the cross talk between Ras and Rho signalling pathways.

Deleted in Liver Cancer (DLC) genes emerged as a novel bona fide tumor suppressor because they are frequently inactivated or down-regulated in a variety of cancers. DLC family proteins (DLC1, DLC2 and DLC3) contain different functional domains, including the catalytic Rho GTPase-activating (RhoGAP) domain. DLC1 has been reported previously to interact with the SH3 (Src homology 3) domain of p120RasGAP (Yang et al., 2009). In this study (Jaiswal et al., *in prep*.) we have investigated that: (i) only the SH3 domain of p120RasGAP, specifically and selectively inhibits the RhoGAP activity of all three DLC isoforms, (ii) unlike classical PxxP motif-recognizing SH3 domains, the interaction mode of p120RasGAP SH3 domain is unique and utilizes different amino acids in order to bind and to displace the catalytic arginine finger of the GAP domain of DLC1.

The GAP catalyzed GTP hydrolysis reaction of Cdc42, RhoA and Rac1 was measured for DLC family proteins by fluorescence based GAP assay (Jaiswal et al., 2012a) in real-time. The observed rate acceleration of DLC1GAP was 332-fold with respect to the intrinsic GTPase activity of Cdc42 and data indicate that DLC1GAP is most efficient for RhoA then Cdc42 and has least activity for Rac1. It also indicates that the

* ***Manuscript in preparation***

DLC family members are inefficient RhoGAPs and their catalytic efficiency is not in the order of efficient RhoGAPs (unpublished).

DLC1 is multimodular protein (Fig. 9). To test whether RhoGAP activity of DLC1 is regulated by its other domains we have first measured the GAP activity of DLC1 f.l., in presence of SAM, START domain and compared with DLC1GAP alone. The obtained data show that the RhoGAP activity of DLC1 full length is rather inhibited. But DLC1GAP activity neither affected by the presence of purified SAM and SATRT domains alone nor in the presence of both together in our experimental conditions. Therefore, we can conclude that the inhibition of DLC1 f.l. could be imposed by the other regions but not by SAM and START domains.

From the study of Yang et al., 2009 new binding partner p120RasGAP-SH3 for DLC1 have been reported and showed that it inhibits its RhoGTPase and growth-suppressing activity (Yang et al., 2009). To test weather this inhibition is conserved for DLC family. We analyzed the GAP activity of DLC family members in presence of SH3 domain of p120RasGAP. The obtained data indicates that the GAP activity of DLC1 drastically reduced upto 83-fold in the presence of SH3 domain and almost abolished the DLAC1GAP mediated GTPase activity of Cdc42. The GAP activity of DLC2 and DLC3 was also reduced drastically in presence of SH3 domain of p12RasGAP. These data clearly indicate that p120RasGAP-SH3 domain mediated inhibition of GAP activity of DLC family members is rather conserved. We also measured the DLC1 GAP activity in presence of different constructs of p120RasGAP all containing SH3 domain: full length, SH2-SH3-SH2 and SH3. All constructs were able to inhibit GAP activity drastically but effect mediated by SH3 domain alone was strongest.

Another issue that we addressed was whether the p120Ras SH3 binding for the GAP domain is specific only for DLC family member or it can bind to any other RhoGAPs. Therefore, we first measured the activity of ten RhoGAPs: Abr, DLC1, GRAF1, MgcRac, Nadrin, OPHN1, p50 and p190 in the absence and presence of p120Ras SH3. The data show that the activity of these RhoGAPs is not affected at all by p120Ras SH3 but only of DLC1 and emphasize that the p120RasGAP SH3 domain-mediated *trans-inhibition* of DLC1 is highly specific. To check if DLC1GAP activity inhibition is caused only by p120RasGAP SH3, we measured the activity of DLC1GAP in presence and absence of eight different SH3 domains: $Crk1^{SH3}$, $Grb2^{SH3-1}$, $Grb2^{SH3-2}$, $Nck1^{SH3-1}$, $Nck1^{SH3-2}$, $Nck1^{SH3-3}$, $p120RasGAP^{SH3}$ and Src^{SH3}. The data indicate that the activity of $DLC1^{GAP}$ is not affected at all by other seven SH3 domains except

p120RasGAP^{SH3} and this emphasize that the p120RasGAP^{SH3} domain-mediated *trans-inhibition* of DLC1 is highly selective.

To analyze the inhibition caused by p20RasGAP SH3 on DLC1GAP activity is via direct or indirect interaction mode we first did the analytical size exclusion chromatography. The formation of stable complex gave the hint that the affinity between DLC1^{GAP} and p120^{SH3} is high. Binding constant (K_d) for p120Ras SH3 and DLC1GAP calculated by isothermal titration calorimetry (ITC) was in high nanomolar range (0.6 μM). This remarkably tight binding of SH3 domain was rather surprising because the affinity of SH3 domains binding is rather found mostly in the micromolar range (Karkkainen et al., 2006). The very few example of high affinity binding of SH3 domains are known as Mona/Gads and SLP-76 (Harkiolaki et al., 2003), C3G and c-Crk (Wu et al., 1995) and Grb2 for its interaction with Wrch1 (Risse et al., revised).

The high affinity of p120RasGAP SH3 domain binding to DLC1 GAP tempted us to explore for the binding mode of SH3-DLC1. The *in silico* observations lead us to conclude that p120RasGAP SH3 binds to DLC1GAP via non-classical mode. To map the speculated binding site(s) on p120RasGAP SH3 that mediates DLC1GAP interaction and inhibit its RhoGAP activity, we did *in silico* study and to identify the structural requirements of the DLC1^{GAP}-p120RasGAP^{SH3} complex. For this the available crystal structure of p120RasGAP^{SH3} (PDB: 2J05) was docked on the crystal structure of DLC1GAP (PDB: 3KUQ) using the program PatchDock (Schneidman-Duhovny et al., 2005). To prove our *in silico* analysis we created point mutations in both SH3 and GAP domains. Point mutations of the residues at N311R, L313A and W319G in SH3 domain of p120Ras and single point mutation in GAP domain (R677A) had varied effects on the interaction between p120RasGAP SH3 and DLC1GAP and also on DLC1 RhoGAP activity inhibition *in vitro*. Mutation of Trp to Gly does not have any effect on interaction and GAP activity while mutation of Asp to Arginine and Leu to Alanine resulted in 60% release in inhibition caused by SH3 wt and also abolish the complex formation. Interestingly while Trp mutation glycine has neither effect on interaction nor GAP activity, mutation of all three not only abolished the complex formation but the release in inhibition was increased upto 90%.

The novel findings of this study shed light on unique molecular mechanisms underlying the DLC inhibitory effects of p120RasGAP and point to an additional level of crosstalk between the Ras and the Rho family GTPases.

8. Summary

The Rho family proteins are identified as the master regulators of the cytoskeleton, control a remarkable diversity of cellular functions from fundamental (e.g. establishment of cell polarity) to highly specialized (e.g. contraction of vascular smooth muscle cells). Rho proteins function as molecular switches that are activated when bound to GTP and inactivated when GTP is hydrolyzed to GDP and P_i. This regulatory cycle is controlled by different regulatory protein families: GEFs and GAPs. Guanine nucleotide exchange factors (GEFs) catalyze the intrinsic nucleotide exchange while GTPase activating proteins (GAPs) stimulate the intrinsic GTP hydrolysis activity of Rho proteins. Abnormal activation of Rho proteins has been shown to play a crucial role in cancer, infectious and cognitive disorders, and cardiovascular diseases. However, there is a series of facts that have led to increase complexity in understanding Rho proteins signaling: (i) The Rho family comprises of 22 genes in humans, encoding at least 25 signaling proteins, of which only RhoA, Rac1 and Cdc42 have been studied in detail so far. The functions of the other uncharacterized members of this protein family await detailed investigation. (ii) To regulate 25 Rho proteins an overwhelming number of their regulatory proteins (>70 GEFs, >70 GAPs and >100 effectors) exists in the human genome. Exactly how these regulators translate specificity is not well understood and majority of GEFs and GAPs in humans so far remain uncharacterized. (iii) Most of the GEFs and GAPs themselves need to be regulated and require activation through the relief of autoinhibitory elements. It is conceptually still unclear how such autoregulatory mechanisms are operated. Understanding the specificity, activation mechanism and the mode of action of these regulatory proteins is a master key for drug development against variety of diseases caused by the involvement of Rho proteins. Hence, the focus of this thesis was to study comprehensively intrinsic and extrinsic functions of Rho family proteins regarding structural determinants, specificity and regulation by GEFs and GAPs.

In this thesis a detailed protocols for the expression and purification of Rho proteins, effector binding domains and the catalytic domains of GEFs and GAPs have been described including the preparation of nucleotide-free and fluorescent nucleotide-bound Rho proteins. Quantitative methods have been developed and established to monitor the intrinsic and GEF-catalyzed nucleotide exchange, the intrinsic and GAP-stimulated GTP-hydrolysis and the effector-interaction with active GTPase for the biochemical analysis of Rho proteins.

To characterize the Rho family proteins regarding their intrinsic properties a comprehensive investigation for fifteen members was performed. This comparative investigation revealed critical differences in the nucleotide binding properties, intrinsic nucleotide exchange and GTP hydrolysis of Rho proteins and shows that even highly related Rho and Rac isoforms have crucial differences in their intrinsic properties. The major finding of the study was highlighting the novel behavior of the two members of the Rho family: RhoD and Rif. Surprisingly, in contrast to conventional Rho proteins RhoD and Rif show faster nucleotide exchange than GTP hydrolysis, which shifts the equilibrium largely towards GTP-bound state. This observation allowed us to conclude that RhoD and Rif does not follow the conventional switch mechanism.

The Dbl family GEFs have been comprehensive investigated *in vitro* and *in silico* for their activity and specificity for their target substrate Rho proteins. Our data show that not all Rho proteins need GEFs and not all Dbl GEFs exhibit GEF activity. The data enable us to conclude that catalytic efficiency of Dbl proteins is proportional to their association reaction and also enable us to classify the 74 Dbl GEFs into subfamilies on the basis of their substrate specificity. We also conducted the comparative investigation of RGS-domain containing Dbl GEFs and show that LARG, PRG, p115 and p190 are specific for the Rho isoforms (RhoA, RhoB and RhoC). N-terminal region of the DH domain of LARG was found to be responsible for its highest GEF activity. The DH domain is sufficient to exhibit catalytic GEF activity and the tandem PH domains of p115 and PRG Dbl GEFs contribute to the DH-mediated nucleotide exchange reaction but not in the association reaction. We also show that the RGS domain inhibit the DH domain mediated GEF activity, which support the proposed models of an intramolecular autoinhibitory mechanism for p115-like DblGEFs.

A tumor suppressor gene, DLC1, is one of the RhoGAP whose regulation was investigated by PKD phosphorylation and p120RasGAP. A novel PKD phosphorylation site of DLC1 was identified within the RhoGAP domain at Ser-807. This phosphorylation has no impact on GAP activity of DLC1 *in vitro* but it negatively regulates DLC1 cellular function. Structural and functional determinants of DLC1RhoGAP *trans inhibition* by the SH3 domain of p120RasGAP were characterized in detail. This study describes a novel and unique interaction mode between GAPs of two distinct GTPase families.

9. Zusammenfassung

Proteine der Rho Familie wurden als Masterregulatoren des Zytoskellets beschrieben. Sie kontrollieren eine Vielzahl von zellulären Funktionen, ausgehend von fundamentalen (z.B. Ausbildung der Zellpolarität) bis hin zu hoch spezialisierten Prozessen (z.B. Kontraktion der vaskulären glatten Muskelzellen). Rho-Proteine (Rho-GTPasen) agieren als molekulare Schalter die im aktivierten Zustand GTP gebunden haben und nach Hydrolyse des GTP zu GDP und P_i inaktiviert sind. Dieser Prozess wird von einer Reihe verschiedener regulatorischer Proteinfamilien kontrolliert: GEFs und GAPs. Guanin-Nukleotid-Austauschfaktoren (guanine nucleotide exchange factors, GEFs) katalysieren den Nukleotidaustausch wohingegen GPTase-aktivierende Proteine (GTPase activating proteins, GAPs) die Rho-Protein-vermittelte Hydrolyse von GTP stimulieren. Eine fehlerhafte Aktivierung von Rho-GTPasen spielt eine entscheidende Rolle bei der Krebsentstehung, bei Infektionskrankheiten und bei kognitiven Störungen sowie bei kardiovaskulären Erkrankungen. Eine Reihe von Fakten führte jedoch zu einer zunehmenden Komplexität beim Verständnis von Rho-GTPasen in der Signaltransduktion: (i) Die Familie der Rho-GTPasen umfasst beim Menschen 22 Gene, die mindestens 25 Proteine kodieren, von denen momentan lediglich RhoA, Rac1 und Cdc42 im Detail untersucht wurden. Die Funktion der bisher nicht charakterisierten Mitglieder dieser Proteinfamilie muss zukünftig genauer untersucht werden. (ii) Für die Regulation dieser 25 Rho-Proteine existiert eine beträchtliche Anzahl von regulatorischen Proteine im menschlichen Genom (>70 GEFs, >70 GAPs und >100 Effektoren). In wieweit eine Spezifität zwischen dieser großen Anzahl von Regulatoren und Rho-Proteinen besteht ist bisher noch unverstanden außerdem ist ein Großteil der humanen GEFs und GAPs bisher noch nicht charakterisiert. (iii) Viele GEFs und GAPs werden reguliert und benötigen für ihre Aktivierung autoinhibitorische Faktoren. Wie dieser Mechanismus der Autoregulation funktioniert ist bisher unklar. Das Verständnis der Spezifität, des Aktivierungsmechanismus und der Wirkungsweise dieser Regulatoren ist der Schlüssel für die Entwicklung von Arzneimitteln gegen eine Vielzahl von Erkrankungen bei denen Rho-Proteine beteiligt sind. Aus diesem Grund war der Schwerpunkt dieser Arbeit eine umfassende Untersuchung der intrinsischen und extrinsischen Funktionen aller Proteine der Rho Familie hinsichtlich ihrer Struktur, Spezifität und Regulation durch GEFs und GAPs.

In dieser Arbeit wurden die Expression und Reinigung von Rho-GTPasen, Effektorbindungsdomänen und katalytischen Domänen von GEFs und GAPs detailliert beschrieben. Die Protokolle beinhalten außerdem die Herstellung von nukleotidfreien Rho-GTPasen und von GTPasen mit fluoreszenzmarkierten Nukleotiden. Im Rahmen der Arbeit wurden Methoden entwickelt, um den intrinsischen und GEF-katalysierten Nukleotidaustausch, die intrinsische und GAP-stimulierte GTP-Hydrolyse und die Effektorinteraktion mit aktiven GTPasen für die biochemische Analysierung von Rho-GTPase zu visualisieren. Um die Familie der Rho-Proteine hinsichtlich ihrer intrinsischen Eigenschaften zu charakterisieren, wurden 15 Mitglieder parallel untersucht. Dieser Vergleich zeigte deutliche Unterschiede in den Nukleotidbindungseigenschaften, dem intrinsischen Nukleotidaustausch und der GPT-Hydrolyse von Rho-Proteinen. Weiterhin wurden große Unterschiede in den intrinsischen Eigenschaften auch zwischen nah verwandten Isoformen von Rho und Rac festgestellt. Die Haupterkenntnis dieser Arbeit war das andersartige Verhalten von zwei Mitgliedern der Rho Familie: RhoD und Rif. Im Gegensatz zu konventionellen Rho-Proteinen zeigten RhoD und Rif einen schnelleren Nukleotidaustausch verglichen mit der GTP-Hydrolyse, wodurch das Gleichgewicht weitgehend auf die Seite des GTP-gebundenen Zustands verschoben wurde. Diese Beobachtung erlaubte die Schlussfolgerung, dass RhoD und Rif nicht über den herkömmlichen Schalter-Mechanismus von GTPasen verfügen.

Die Dbl-GEFs wurden umfassend *in vitro* und *in silico* hinsichtlich ihrer Aktivität und Spezifität zu Rho-Proteinen untersucht. Unsere Ergebnisse zeigten, dass nicht alle Rho-GTPasen GEFs für ihre Aktivierung benötigten und, dass nicht alle Dbl-GEFs eine GEF-Aktivität aufwiesen. Ausgehend von diesen Ergebnissen lag die Schlussfolgerung nahe, dass die katalytische Effizienz von Dbl-Proteinen proportional ist zu ihrer Assoziationsreaktion ist. Weiterhin erlaubten diese Ergebnisse eine Klassifizierung der 74 Dbl-GEFs in Subfamilien anhand ihrer Substratspezifität.

Im Rahmen dieser Arbeit wurden weiterhin Dbl-GEFs mit RGS-Domäne untersucht. Die Ergebnisse zeigten, dass LARG, PRG, p115 und p190 spezifisch für die Rho Isoformen (RhoA, RhoB und RhoC) sind. Die N-terminale Region der DH-Domäne von LARG besaß hierbei die höchste katalytische Nukleotidaustauschaktivität. Die DH-Domäne war ausreichend für die katalytische GEF-Aktivität und die Tandem PH-Domänen von p115 und PRG beeinflussten entscheidend die DH-vermittelten Nukleotidaustauschreaktion jedoch nicht die Assoziationsreaktion. Weiterhin wurde gezeigt, dass die RGS-Domäne die durch die DH-Domäne vermittelte GEF-Aktivität

inhibiert. Dies wiederum unterstützt das vorgeschlagene Modell eines intramolekularen autoinhibierenden Mechanismus für p115-ähnliche Dbl-GEFs.

Das Genprodukt des Tumorsupressorgens DLC1, ist ein Protein der Rho-GAP Familie und wurde hinsichtlich seiner Regulation durch p120RasGAP und PKD Phosphorylierung untersucht. Eine neue PKD Phosphorylierungsstelle von DLC1 innerhalb der RhoGAP Domäne (Ser-807) konnte identifiziert werden. Diese Phosphorylierung hatte keinen Einfluss auf die GAP-Aktivität von DLC1 *in vitro* aber regulierte die zelluläre Funktion von DLC1 negativ. Strukturelle und funktionelle Faktoren der DLC1RhoGAP *trans* Inhibition durch die SH3 Domäne von p120RasGAP wurde im Rahmen der Arbeit detailliert charakterisiert. Diese Untersuchungen beschreiben einen neuen und einzigartigen Interaktionsmechanismus zwischen GAPs von zwei verschiedenen GTPase Familien.

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11. Abbreviations

AbetaPP	Amyloid beta protein precursor
ANK	Ankrin Repeat
BAR	Bin/amphiphysin/Rvs
CaaX	Cysteine (C)-two aliphatic residues (aa)-variable amino acid (X)
CC	Coiled-coil region
Cdc42	Cell division cycle 42
Chp	Cdc42 homologous protein
CZH	CDM-zizimin homology
Dbl	Diffuse B-cell lymphoma
Dbs	Dbl's big sister
DH	Dbl homology
DHL	DH-like
DHR1	Dock-homology region regulatory
DOCK	Dedicator of cytokinesis
F	Farnesylation
F-actin	Filamentous actin
FCH	Fes/CIP4 homology
FFTase	Farnesyltransferase
GAP	GTPase activating protein
GDP	Guanine diphosphate
GDI	GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GG	Geranyl-geranylation
GGTase I	Geranylgeranyltransferase I
GNBPs	Guanine nucleotide binding proteins
GPCRs	G-protein coupled receptors
GRK2	G protein receptor kinase 2
GTP	Guanine triphosphate
GTPases	Guanosine triphosphatases
HTGPs	Hetrotrimeric G proteins
HRas	Harvey-rat sarcoma
HVR	Hypervariable regior
IRS-1	Insulin receptor substrate-1
IIP5	Inositol 5-phosphatase catalytic
KRas	Kirsten-rat sarcoma
LARG	Leukemia-associated Rho guanine exchange factor

NLS	Nuclear localization signal
P	Palmotylation
PH	Pleckstrin homology
PRG	PDZ (postsynaptic density-95, discs large, and zona occludens)-RhoGEF
Rac1	Ras related C3 botulinum toxin substrate 1
Ras	*Rat sarcoma*
RA	Ras-associating
Rho	*Ras homolog*
RhoBTB	Rho Broad-Complex, Tramtrack and Bric-a-brac
SAM	Sterile α motif
SH2	SSrc homology 2
SH3	Src homology 3
SIMIBI	Signal recognition particle, MinD and BioD
Sos1	Son of Sevenless 1
START	StAR (steroidogenic acute regulatory)-related lipid transfer
SWAP70	Switch-associated protein 70
TC10	Teratocarcinoma 10
TCL	TC10-like
TRAFAC	Translation Factors
Wnt	Wingless/Int
Wrch-1	Wnt-reglated Cdc42 homolog-1

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13. Curriculum Vitae

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Publications

[1] **Jaiswal, M.**, Fansa, E. K., Dvorsky, R., Ahmadian, M. R. (2012). *New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm*. **Biol Chem**. (in press)

[2] **Jaiswal, M.**, Dubey, B.N., Koessmeier, K.T., Gremer, L., and Ahmadian, M.R. (2012). *Biochemical assays to characterize Rho GTPases*. **Methods Mol Biol** *827*, 37-58.

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[4] Scholz, R.P., Gustafsson, J.O., Hoffmann, P., **Jaiswal, M.**, Ahmadian, M.R., Eisler, S.A., Erlmann, P., Schmid, S., Hausser, A., and Olayioye, M.A. (2011). *The tumor suppressor protein DLC1 is regulated by PKD-mediated GAP domain phosphorylation*. **Exp Cell Res** *317*, 496-503.

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[8] **Jaiswal, M.,** Dvorsky, R., Koessmeier, K., Ahmadian, M.R., *An ensemble approach for deciphering the molecular and functional basis of GTPase activating proteins of the Rho family*.

14. Enclosures

1. **Biochemical assays to characterize Rho GTPases**
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2. **New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm**
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3. **Mechanistic insights into specificity, activity, and regulatory elements of the regulator of G-protein signaling (RGS)-containing Rho-specific guanine nucleotide exchange factors (GEFs) p115, PDZ-RhoGEF (PRG), and leukemia-associated RhoGEF (LARG)**
   Jaiswal, M.* , Gremer, L.*, Dvorsky, R*., Haeusler, L.C., Cirstea, I.C., Uhlenbrock, K., and Ahmadian, M.R.
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4. **Deciphering the molecular and functional basis of Dbl family proteins: a meta-analysis approach**
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5. **The tumor suppressor protein DLC1 is regulated by PKD-mediated GAP domain phosphorylation.**
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Chapter 3

Biochemical Assays to Characterize Rho GTPases

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Abstract

Rho GTPases act as tightly regulated molecular switches governing a large variety of critical cellular functions. Their activity is controlled by two different biochemical reactions, the GDP/GTP exchange and the GTP hydrolysis. These very slow reactions require catalysis in cells by two kinds of regulatory proteins. While the guanine nucleotide exchange factors (GEFs) activate small GTPases by stimulating the exchange of bound GDP for the cellular abundant GTP, GTPase-activating proteins (GAPs) accelerate the intrinsic rate of GTP hydrolysis by several orders of magnitude, leading to their inactivation. There are a number of methods that can be used to characterize the specificity and activity of such regulators to understand the effect of binding on the protein structure and, ultimately, to gain insights into their biological functions. This chapter describes (1) detailed protocols for the expression and purification of Rho GTPases, of effector-binding domains, and catalytic domains of GEFs and GAPs; (2) the preparation of nucleotide-free and fluorescent nucleotide-bound Rho GTPases; and (3) methods for monitoring the intrinsic and GEF-catalyzed nucleotide exchange, the intrinsic and GAP-stimulated GTP hydrolysis, and the effector interaction with active GTPase (three alternative approaches).

Key words: Fluorescence spectroscopy, GAP, GEF, GTPase, Guanine nucleotide, Mant, Protein–protein interactions, Rho, Tamra, Effector

1. Introduction

Rho family GTPases act as tightly regulated molecular switches governing a variety of critical cellular functions (1–5). Their activity is controlled by two biochemical reactions, the GDP/GTP exchange and the GTP hydrolysis, which can be catalyzed by two kinds of regulatory proteins (6). While the guanine nucleotide exchange factors (GEFs) activate Rho GTPases by stimulating the slow exchange of bound GDP for the cellular abundant GTP,

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GTPase-activating proteins (GAPs) accelerate the slow intrinsic rate of GTP hydrolysis by several orders of magnitude, leading to inactivation. The formation of the active GTP-bound state of the GTPase is accompanied by conformational changes mainly at two regions (called switch I and II) that provide a platform for a selective interaction with a multitude of downstream effectors, which in turn initiate downstream signaling (6–8).

Our understanding of Rho GTPase regulation and signaling is becoming increasingly complex since more than 69 GEFs, 80 GAPs, and 90 effectors are considered to be potential interacting partners of the 22 mammalian members of the Rho family (6, 9–11). Only a sparse number of such intermolecular interactions have been primarily investigated in vitro with solid-phase methods like radioactive ligand overlay, pull-down assays, or yeast two-hybrid studies. These methods are often not sufficient to determine the specificity of regulation and to quantify the activity of recombinant proteins. However, many of the potential interactions defined by these methods require a more detailed analysis of their kinetics by appropriate real-time methods. To obtain a detailed picture of the molecular switch function of Rho GTPases and their interaction with regulators and effectors, fluorescent guanine nucleotides are often ideally suited to fulfill these criteria as it is known that they do not grossly disturb the biochemical properties of the GTPase and that the fluorescence reporter group is sensitive to changes in the local environment to produce a sufficiently large fluorescence change (12–14). Furthermore, the reporter group is often sensitive to the interaction with partner proteins that are able to bind in its neighborhood.

This chapter describes the application of two different fluorescently labeled guanine nucleotides in the biochemical analysis of Rho GTPases (see Fig. 1), which can be used to determine the binding affinities of regulators and effectors as well as to evaluate the activities of GEF-catalyzed nucleotide exchange and GAP-stimulated GTP hydrolysis, respectively.

2. Materials

2.1. Bacterial Strains

Different *Escherichia coli* strains BL21(DE3), BL21(DE3) codon plus RIL, BL21(DE3) pLysS, BL21(DE3) Rosetta (Novagen) are used to recombinantly express eukaryotic genes and gene fragments.

2.2. Chemicals and Reagents

1. Isopropyl-β-D-thiogalactopyranoside (IPTG).
2. 6 M guanidinium hydrochloride.

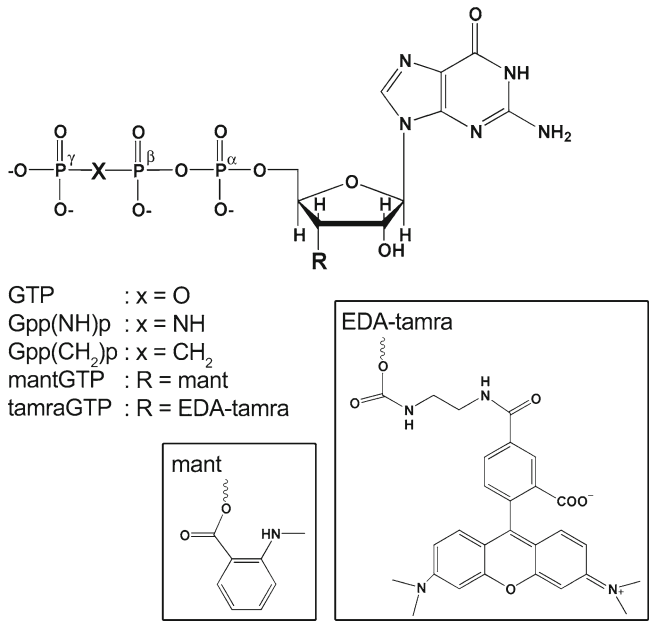

Fig. 1. Chemical structures of the guanosine nucleotide derivatives used in this chapter. Unlabeled fluorescent nucleotides contain an OH group at the position R.

3. Enzymes: Thrombin (Serva), PreScission protease (GE Healthcare), TEV protease (Invitrogen), agarose bead-coupled alkaline phosphatase (Sigma–Aldrich), soluble alkaline phosphatase (Roche Diagnostics), snake venom phosphodiesterase (Sigma–Aldrich).
4. Ponceau Red: 0.1% Ponceau Red S (w/v), 5% acetic acid (v/v) in double-distilled water.
5. 500 mM ethylendiamine-*N*, *N*, *N′, N′*-tetraacetic acid (EDTA), pH 8.0, adjusted with 1 N NaOH.
6. Nitrocellulose membrane.
7. Amicon Ultra centrifugal filter units (Millipore) with molecular mass cutoff of 5–100 kDa for concentrating proteins.
8. Bottle-top filter units with 0.2-μm cutoff, 500 mL, for filtering buffer and solutions.

2.3. Nucleotides

The nucleotides used in biochemical assays are adenosine 5′-triphospahte (ATP), guanosine 5′-diphosphate (GDP), and guanosine 5′-triphosphate (GTP) (Pharma Waldhof; 10 mM in deionized water, pH 7.5), the nonhydrolyzable GTP analog β, γ-methyleneguanosine 5′-triphosphate [Gpp(CH_2)p] and guanosine 5′-[β,γ-imido]triphosphate (GppNHp) (Sigma–Aldrich and Jena Biosciences).

Two different fluorescence reporter groups, *N*-methylanthraniloyl (mant) (12) and tetramethylrhodamine (tamra) (14, 15), attached to the 2′(3′)-hydroxyl group of the ribose moiety of the guanine nucleotides (GDP, GTP, and GppNHp) (see Fig. 1), are used both to monitor protein–ligand as well as protein–protein interactions and to measure catalytic activities of regulatory proteins. The fluorescent nucleotides mantGDP, mantGTP, mantGppNHp (10 mM solution in deionized water, pH 7.5), and tamraGTP (2 mM solution in deionized water, pH 7.5) are synthesized as described in ref. 13 and can be purchased from Jena Biosciences.

2.4. Buffers and Media

Prepare all solutions in double-distilled water at room temperature (25°C), unless indicated otherwise. All buffers should be filtered and degassed.

1. Terrific broth (TB) medium: 12 g/L bacto-tryptone, 24 gL yeast extract, 0.4% (v/v) glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4.
2. Lysis buffer: 30 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 3 mM DTT, 100 mM NaCl, lysozyme (0.5 mg/mL), DNAse I (10 µg/mL), complete EDTA-free protease inhibitor cocktail (Roche) (1 tablet per 200 mL).
3. Wash buffer: 30 mM Tris–HCl, pH 7.5, 50 mM Nacl, 5 mM $MgCl_2$, 3 mM DTT.
4. Standard buffer: 30 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 3 mM DTT, 100 mM NaCl.
5. High-salt ATP buffer: Standard buffer containing 400 mM KCl and 1 mM ATP.
6. Glutathione (GSH) elution buffer: Standard buffer containing 20 mM reduced glutathione (Merck), pH 7.5 (adjusted with 1 N NaOH).
7. Exchange mix (10×): 2 M $(NH_4)_2SO_4$, 10 mM $ZnCl_2$.
8. HPLC buffer: 100 mM K_2HPO_4/KH_2PO_4, pH 6.5, 10 mM tetrabutylammonium bromide, 7.5–25% (v/v) acetonitrile.
9. GEF buffer: 30 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 3 mM DTT, 10 mM K_2HPO_4/KH_2PO_4, pH 7.4.
10. GAP buffer: 30 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 3 mM DTT, 10 mM K_2HPO_4/KH_2PO_4, pH 7.4.
11. Effector buffer: 30 mM Tris–Cl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 3 mM DTT.

2.5. Chromatography Columns

Following columns are used for protein purification and analysis: Reversed-phase C-18 HPLC column Ultrasphere ODS, 5 µm; 250×4, 6 mm (Beckman Coulter), guard column Nucleosil 100-5-C18, 5 µm (Bischoff Chromatography); glutathione sepharose

4B FF column (GE Healthcare); Superdex 75 or 200, 16/60, or 26/60 columns (GE Healthcare) (column dimensions are given as 16- or 26-mm diameter and 60-cm length); NAP 5 column (GE Healthcare).

2.6. Instruments

1. Shaker incubator (Infors HT).
2. Sonicator (Bandelin electronics).
3. M-110S laboratory microfluidizer processor (Microfluidics).
4. Centrifuge Avanti J-20 XP with 6-L rotor JLA-8.1000 and rotor JA 25.50 (Beckman Coulter) or equivalent.
5. ÄKTA prime and purifier (GE Healthcare).
6. UV–Vis spectrometer (Biophotometer, Eppendorf).
7. HPLC instrument (Beckman Gold, Beckman Coulter).
8. Fluorescence spectrometer (Perkin-Elmer, LS50B; FluoroMax-4, Horiba).
9. Stopped-flow instrument (Applied Photophysics SX18MV or Hi-Tech SF-61 DX2).
10. Quartz cuvettes (Suprasil 108.002F-QS, Hellma).

2.7. Software

Program packages of Grafit (Erithacus Software), Origin (OriginLab), and Sigmaplot (Systat Software Inc) are used for the evaluation of the data.

3. Methods

3.1. Gene Expression and Bacterial Culture Conditions

High quality (>95% purity) and quantity (>10 mg) of purified proteins are mandatory prerequisites for the investigation of relationships between protein structure and function. Recombinant expression systems and the development of a variety of fusion tags have dramatically facilitated purification. Nevertheless, the choice of the right purification strategy is still a matter of trial and error and has to be elaborated for each individual protein.

To optimize the synthesis of the protein of interest in *E. coli*, various culture conditions should be examined, including the IPTG concentration as the inducer of the *lac*-promoter-controlled gene expression, the optical density (OD_{600}) at the time of induction, and the culture temperature and expression time post induction. Culture condition tests varying these parameters should be performed in small-scale studies prior to upscaling the cultures for preparative protein expression. To improve a maximal yield of the desired protein, we alternatively use, besides the *E. coli* strain BL21 (DE3), strains containing additional plasmids, such as pLysS

(to improve bacterial lysis and for the expression of toxic proteins) and Codon plus RIL or Rosetta (to improve the codon usage).

1. Grow 30–250 mL of precultures of the desired *E. coli* strain in TB medium in a 150–1,000-mL flask overnight at 37°C (see Note 1).
2. Fill 5-L Erlenmeyer flasks with 2.5 L of TB medium. Inoculate each flask with 25 mL of an overnight preculture (see Note 2). Place the inoculated culture flasks in an environmental shaker and let them grow at 37°C with shaking at 160 rpm.
3. When the logarithmic growth phase is reached (OD 0.4–0.8), lower the temperature to the previously optimized expression condition (usually, 18–30°C), add IPTG (usually, 0.05–0.5 mM) (see Note 3), and incubate the culture usually overnight and in rare cases for only 3–6 h.
4. Transfer the cells to 1,000-mL centrifuge bottles and harvest the cells by centrifugation at 5,000 × *g* for 15 min at 4°C using a 6-L rotor if available. Repeat this step several times if the culture volume exceeds the capacity of the available rotor.
5. Wash the bacterial pellet in each rotor bottle with 20 mL of wash buffer. Combine the resuspended cell pellets into a smaller rotor bucket and centrifuge again at 5,000 × *g* for 20 min at 4°C (see Note 4).
6. Discard the supernatant and determine the weight of the bacterial pellets (the tare of the centrifuge beaker should be known before). Resuspend the pellets in wash buffer (3 mL/g bacterial pellet) or any other appropriate buffer that is able to solubilize and stabilize the desired protein and distribute in aliquots in 50-mL plastic tubes.
7. Store aliquots at –20°C (see Note 5).

3.2. Bacterial Lysis

The efficient bacterial lysis is an important prerequisite for the complete recovery of the recombinant protein. Cell walls of bacteria must be disrupted in order to allow access to intracellular components. Different methods have been evolved to achieve this goal, which vary considerably in the severity of the disruption process, reagents needed, and the equipment available. Besides enzymatic methods, e.g., lysozyme treatment, which is suitable for analytical scales and not always reproducible, there are several mechanical methods available, including bead milling with glass beads, the "cell disruption bomb," high shear mechanical methods like the "French press" and the "microfluidizer," or sonication with ultrasound to gently disrupt bacterial cell walls. We commonly use the latter two methods, which are efficient and fairly quick.

3.2.1. Bacterial Lysis by Sonication

1. Equip the cell sonicator with a titanium horn of 3- to 19-mm diameter (depending on the culture volume to be disrupted) and clean it before use with 70% ethanol.
2. Transfer the defrosted bacterial suspension from all aliquots to a beaker of suitable size and place it on ice.
3. Place the sonicator horn about 0.5–1 cm immersed into the suspension and stir the suspension on a magnetic stirrer.
4. Start the sonication procedure by increasing the output control (5–10 W each) in 10-s intervals starting with low, 30 W, to reach finally 95 W. Repeat this procedure several times (8–12) and always wait for 30 s in between to prevent overheating of the sample. For the latter reason, the beaker with the bacterial solution also needs to be stored on ice during the whole procedure (see Notes 6–8).

3.2.2. Bacterial Lysis Using a Microfluidizer

The microfluidizer is an instrument that uses high pressure to squeeze the bacterial solution through an interaction chamber containing a narrow channel, thereby generating high shear forces that pull the cells apart. The system permits controlled cell breakage and does not need addition of detergent or higher ionic strength. Since heat is generated during this process, an interaction chamber needs to be cooled.

1. Wash the instrument extensively with water. For a final wash step, use the standard buffer used for the protein purification (see Note 9). Pour the defrosted bacterial suspension into the instrument's reservoir and turn on the instrument (see Note 10). Direct the flow on the instrument's outlet toward the wall of a beaker to prevent foam formation.
2. Prevent intake of air on the inlet as this also produces foam and lead to protein denaturation. For this, turn off the instrument before air enters the instrument's inlet. Wash with a small volume of standard buffer and switch the instrument on again. Stop again before air enters the inlet. By repeating this step 2–3 times, nearly all of the bacterial suspension is processed.
3. If necessary, flush the bacterial suspension 2–3 times through the instrument until a color change from milky to slightly more translucent is observed.
4. Wash the instrument extensively with water and finally with 2-propanol. Store it in this alcohol.

3.3. Protein Purification

3.3.1. Purification Steps as GST Fusion

1. Centrifuge the bacterial lysate typically derived from 5 to 15 L of cell culture to sediment insoluble components at 35,000–100,000 $\times g$ at 4°C for 40 min. If possible, centrifuge at 100,000 $\times g$ to remove insoluble cell fragments quantitatively.

If a high-speed rotor/centrifuge is not available, a minimal force of 35,000 × *g* might also be sufficient.

2. Equilibrate a GSH sepharose column (10–25 mL bed volume) with approximately 3–4-column volumes of standard buffer until a stable baseline absorption monitored at 280 nm is achieved (see Note 11).
3. After centrifugation, apply the cleared bacterial lysate on the GSH sepharose column (4 mL/min, if using fast flow material). After all lysate is applied, wash with standard buffer until the baseline at 280 nm is reached again. Wash with 100–200 mL of high-salt ATP buffer (see Note 12).
4. Wash with at least 100 mL of standard buffer for removal of KCl and ATP until the original baseline level is achieved.
5. Elute GST fusion proteins from the GSH sepharose column with 100–150 mL of GSH elution buffer and collect the eluting GST fusion protein in 5–10-mL fractions (see Note 13).
6. Pool the GST fusion protein-containing fractions after analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining following standard procedures. Regenerate the column with 50 mL of aqueous 6 M guanidinium hydrochloride and wash with 100–150 mL of standard buffer afterward.

3.3.2. Removal of the GST Tag

The GST tag that helps to purify a recombinant protein from crude cell extracts should be removed when the protein shall be used for structural or biochemical analysis. Usually, expression vectors have protease-specific cleavage sites inserted between the coding sequence for the fusion tag and the multiple cloning site. The corresponding fusion protein, thus, can be processed and cut with the appropriate protease and finally the fusion tag can be removed by further chromatographic purification steps.

1. Cleave fusion proteins (≥1 mg/mL) in batch by applying 1–2 U of the appropriate protease (thrombin, TEV, or PreScission depending on the available cleavage site in the vector) per mg of GST fusion protein and incubate for 4–20 h at 4°C. Take a sample of 10 µL from the reaction batch after 4 h and after overnight incubation and analyze by SDS-PAGE and Coomassie blue staining using standard methods to monitor progress of the cleavage.
2. Further purification and removal of protein impurities or small components including reduced GSH is achieved by size-exclusion chromatography (gel filtration) on the scale of 16/60 or 26/60 columns using Superdex 75 or Superdex 200 material (see Note 14). Equilibrate the column with at least one-column volume (130 mL for 16/60 Superdex or 340 mL for

26/60 Superdex) of standard buffer. Load a 1–5-mL sample of the concentrated protein (≤20 mg/mL).

3. Collect in 3–5-mL fractions and withdraw 10-µL samples for analysis by SDS-PAGE and Coomassie staining. Identify the desired protein by its molecular mass and pool the corresponding fractions.
4. In cases where the desired protein (after protease cleavage of the GST tag) has a similar molecular mass as GST, the desired protein cannot be simply removed by gel filtration. Then, the tag has to be removed by a second chromatography on a GSH sepharose column. For that, apply the cleavage reaction on a GSH sepharose column equilibrated with standard buffer and collect the flow through containing the desired protein in 5–10-mL fractions, and then apply standard buffer until the absorbance at 280 nm reaches the baseline level (see Note 15). To regenerate the column, wash with GSH elution buffer to elute the bound GST tag. Withdraw 10-µL samples of the flow-through fractions and analyze by SDS-PAGE and Coomassie staining.
5. Pool and concentrate the desired protein to 10–20 mg/mL.
6. Snap freeze purified proteins in 50–500-µL aliquots in liquid nitrogen and store at –20°C or preferably at –80°C (see Note 16).

3.4. Preparation of Nucleotide-Free Forms of Rho GTPases

Preparation of nucleotide-free GTPase is carried out in two steps: (1) The GTPase-bound GDP is degraded by agarose bead-coupled alkaline phosphatase and replaced by Gpp(CH_2)p (a nonhydrolysable GTP analog, which is resistant to degradation by alkaline phosphatase but sensitive to phosphodiesterase). (2) After the GDP is completely degraded, phosphodiesterase from snake venom is added to the solution of the Gpp(CH_2)p-bound GTPase to cleave this nucleotide to GMP and P_i.

1. Add a 1.5 molar excess of Gpp(CH_2)p to 1 mg of GDP-bound GTPase in standard buffer, apply to the reaction batch the 10× exchange mix, thereby diluting the latter to a 1× concentration, mix rapidly, and withdraw a sample for isocratic ion-pair reversed-phase HPLC analysis on a C_{18} column of the GDP and Gpp(CH_2)p content using HPLC buffer containing 7.5% acetonitrile; for this, dilute the withdrawn sample to 20–100 µM GTPase.
2. Add 0.5–1 U of agarose bead-coupled alkaline phosphatase to the reaction setup, mix rapidly, and incubate at 4°C for 2–16 h (depending on the GTPase used). Analyze the GDP degradation regularly by HPLC determination of the GDP content as described in step 1.
3. After the GDP is completely degraded, centrifuge the suspension at 1,500 × *g*, 4°C, for 2 min to remove the bead-coupled

alkaline phosphatase. Repeat this process two to three times to remove quantitatively all traces of alkaline phosphatase-coupled beads (see Note 17).

4. Add 0.002 U of snake venom phosphodiesterase per mg of GTPase to cleave Gpp(CH_2)p to GMP, guanosine, and P_i. Monitor Gpp(CH_2)p degradation by HPLC as described in step 1.
5. When degradation of Gpp(CH_2)p is complete, inactivate phosphodiesterase by snap freezing in liquid nitrogen. Quickly defrost by warming up the vials in the hands and freeze again in liquid nitrogen; repeat these steps two times and store the protein solution in aliquots at –80°C (see Note 18).

3.5. Preparation of mantGDP-Bound GTPases

Loading of nucleotide-free forms of GTPases with fluorescently labeled nucleotides can be achieved by simply mixing both components followed by a small-scale size-exclusion chromatography on a desalting column. The steps described below are usually necessary for the preparation of GTPases bound to fluorescent GDP analogs.

1. Equilibrate an NAP5 column with 2–3-column volumes of standard buffer.
2. Mix 0.5 mg of a nucleotide-free GTPase (e.g., 50 µL from a 0.5 mM solution) with a 1.5-fold molar excess of mantGDP (e.g., 3.75 µL from a 10 mM stock solution).
3. Apply the complete sample volume on the NAP5 column and allow the sample to enter the gel bed completely.
4. Apply standard buffer to achieve a total applied volume of 500 µL and allow the buffer to enter the gel bed completely (e.g., for the upper example, add 446.25 µL).
5. Add 1 mL of standard buffer and collect fractions (2 drops per fraction).
6. Analyze the protein content of the fractions by dotting 2 µL from each fraction on a nitrocellulose membrane and subsequently staining with Ponceau S. This qualitative test is just to determine which fractions do contain the protein of interest for subsequent pooling.
7. Pool protein-containing fractions and determine the concentration of mantGDP bound to the protein by HPLC using an HPLC buffer containing 20–25% acetonitrile.
8. Store the protein in aliquots at –80°C.

3.6. Preparation of GppNHp-Bound and mantGppNHp-Bound GTPases

GppNHp-bound and mantGppNHp-bound GTPases are prepared using soluble alkaline phosphatase, which degrades the bound GDP

as described in Subheading 3.4, steps 1–3, and Subheading 3.5, steps 7–8.

3.7. Intrinsic and GEF-Catalyzed Nucleotide Exchange Reactions

Different procedures are available for the investigation of the nucleotide exchange on small GTPases. The dissociation of a protein-bound nucleotide can be easily determined in real time by fluorescence spectroscopy using a fluorescent GDP analog. Usually, mant derivatives of guanosine nucleotides, which are coupled at the 2′(3′) hydroxyl group of the ribose, are used.

In principle, each nucleotide-binding protein has a defined intrinsic rate of GDP release, which is often too slow to be physiologically relevant. Thus, GEFs operate on these small GTPases and catalyze the generation of the active GTP-bound state from the inactive GDP-bound form. This process is often a result of the GEFs themselves being activated or recruited to the vicinity of the corresponding GTPase in response to extracellular signaling events.

3.7.1. Measurement of Slow Reactions

Specificity and activity of GEFs can be analyzed qualitatively by comparison of intrinsic and GEF-stimulated fluorescence measurements. Usually, this is performed in a fluorescence spectrometer, since the timescale of these reactions is slow (>1,000 s). Here, the bacterially expressed and highly pure (>90% homogeneity) recombinant mantGDP-loaded GTPases are mixed with an excess of a pure nonfluorescent nucleotide solution in a cuvette. The decrease of the mant-fluorescence signal is monitored with a fluorescence spectrometer. GEF and also GAP assays do not need posttranslationally modified GTPases. Thus, proteins and protein domains produced in *E. coli* can be used (see Note 19). Cleared cell lysate is not suitable in this assay for several reasons: (1) the protein concentration may not be sufficient; (2) the protein of interest may exist in complex with other proteins and may thus not be freely accessible; (3) the activity of other regulators may interfere with the assay. The latter aspect should also be considered with solid-phase-enriched proteins (using GST- or His-tagged proteins) or subcellular fractionated samples.

1. Preincubate the solution of 0.1 μM mantGDP-bound GTPase (see Subheading 3.5) in a fluorescence cuvette (see Note 20) in degassed GEF buffer in a final volume of 600 μL and at 25°C for at least 5 min.
2. Record the mant-fluorescence signal in a fluorescence spectrometer applying an excitation wavelength of 366 nm and an emission wavelength of 450 nm, an integration time of at least 2 s, and a recording time for each data point of 20 s.
3. If the fluorescence signal is stable, add 1.2 μL of a 10 mM nonfluorescent GDP solution (20 μM final GDP concentration) and mix rapidly with a pipette to start the reaction.

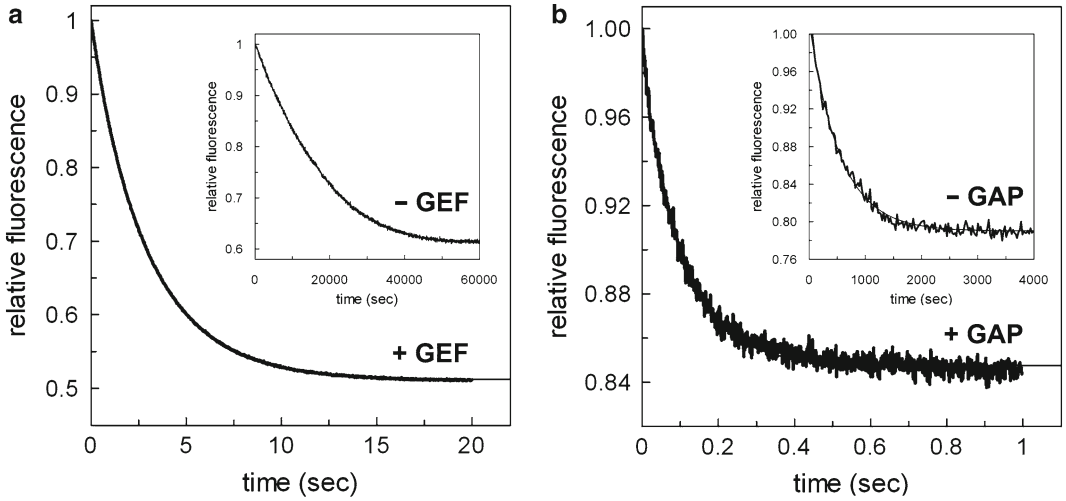

Fig. 2. Monitoring the nucleotide exchange and hydrolysis reactions. (**a**) A sample containing 0.1 μM RhoA·mantGDP was rapidly mixed with 20 μM GDP and 10 μM PDZ-RhoGEF in a stopped-flow instrument to monitor the GEF-catalyzed mantGDP dissociation from RhoA in real time. The intrinsic mantGDP dissociation (in the absence of the GEF protein) was measured under the same conditions in a fluorescence spectrometer (inset). The data are fitted as single-exponential decay of the curve and the observed rate constants (k_{obs}) obtained were 0.000069 s^{-1} for the intrinsic reaction and 0.34 s^{-1} for the GEF-catalyzed reaction that indicate a highly efficient catalysis of about 4,928 fold. (**b**) A sample containing 0.2 μM Cdc42·tamraGTP was rapidly mixed with 2 μM p50Cdc42GAP in a stopped-flow instrument to follow the GAP-stimulated tamraGTP hydrolysis reaction in real time. The intrinsic tamraGTP hydrolysis (in the absence of the GAP protein) was measured under the same conditions in a fluorescence spectrometer (inset). The data was fitted as single-exponential decay to obtain the k_{obs} values of 0.0018 s^{-1} and 9.66 s^{-1} for the intrinsic and the GAP-stimulated reaction, respectively, indicating a rather efficient catalysis of about 5,367 fold.

4. Normally, an exponential decrease in fluorescence occurs and can be monitored over the time course of the reaction (2–72 h), which is due to the mantGDP release into the aqueous solution.
5. When there is no further change in fluorescence, add 24 μL of a 0.5 M EDTA solution to adjust a final concentration of 20 mM and monitor the reaction for additional 10 min. This reveals whether the nucleotide dissociation reaction is completed at all.
6. Fit the data single exponentially with, for example, the Grafit program to provide the dissociation (k_{off}) rates, which are in case of small GTPases usually around 10^{-3} to 10^{-5} s^{-1} (Fig. 2a, inset).

3.7.2. Measurement of Fast Reactions

For fast GEF-catalyzed nucleotide dissociation reactions, the time resolution of a fluorescence spectrometer is insufficient. Instead, a stopped-flow instrument is routinely used for the analysis of rapid kinetics as obtained by quantitative GEF-stimulated nucleotide exchange reactions. Here, equal volumes of two different twofold concentrated samples are automatically shot into a mixing chamber, where the fluorescence changes can be detected directly after

the rapid mixing (death time around 2–5 ms) (see Note 21). Five to eleven identical measurements are recorded and averaged in order to obtain a higher accuracy.

1. Wash the drive syringes of the instrument several times with 5–10 mL of GEF buffer and adjust the temperature to 25°C.
2. Prepare two different samples in degassed GEF buffer at room temperature (~25°C) and a final volume of 1,000 µL: (1) one sample contains 0.2 µM of mantGDP-bound GTPase; (2) the other sample contains the GEF protein (at varying concentrations of 2–1,000 µM depending on the activity and affinity of the GEF for the respective GTPase) plus 40 µM GDP (200-fold excess above mantGDP) (see Note 22).
3. Load each sample into one of the two drive syringes of the stopped-flow instrument.
4. Set the excitation wavelength for the mant nucleotides to 366 nm and detect the fluorescence with a cutoff filter mounted in front of a photomultiplier (408 nm for mant nucleotides).
5. Start the measurement with the supplied stopped-flow software which initiates the pushing of the two syringes containing the samples to the sample cell. At this stage, both samples join together and rapidly mix to a final volume of about 50–75 µL. Repeat the mixing and fluorescence recording event up to 11 times until all volume in the drive syringe reservoir is consumed.
6. Evaluate obtained data by single-exponential fitting with scientific software, e.g., the Grafit program, to obtain the observed rate constant (k_{obs}) for the respective concentration of the GEF protein (see Fig. 2a).

3.8. Intrinsic and GAP-Stimulated GTP Hydrolysis Reaction

The intrinsic and GAP-stimulated GTP hydrolysis reaction of GTPases can be measured by different methods. A generally useful and accurate method is HPLC, by which concentrations of GDP and GTP can be determined from the area of the elution peaks. The relative GTP content determined as the ratio [GTP]/([GTP]+[GDP]) is used to describe the reaction progress as described (14). Measured by HPLC, intrinsic GTP hydrolysis rates of small GTPases of the Rho family are about fivefold faster than that of Ras proteins (e.g., 0.028 min^{-1} for H-Ras) (16, 17). A different approach, which is less material and time consuming, is the real-time measurement of tryptophane fluorescence with an excitation wavelength of 295 nm and an emission wavelength of 350 nm, by which the GTPase reaction rates can be conveniently measured. The Ras(Y32W) mutant provided a large increase in fluorescence signal upon hydrolysis of GTP to GDP, which has been used to study the mechanism of the intrinsic GTPase reaction (17).

Whether this approach is useful for the members of the Rho family remains to be investigated.

Unlike mant, tamra is a powerful fluorescence reporter group to study in real time the GTP hydrolysis of Rho GTPases in the presence and absence of their GAPs (15). The intrinsic and GAP-stimulated tamraGTP hydrolysis reaction of RhoGTPases can be detected by conventional fluorescence spectrometric and stopped-flow measurements, showing a significant decrease in the fluorescence signal.

3.8.1. Measurement of Slow Reactions

In contrast to other fluorescent nucleotide derivatives, including the mant nucleotides, tamraGTP (a ribose hydroxyl-substituted tetramethylrhodamine derivative of GTP) enables us to measure the intrinsic and GAP-stimulated GTPase reactions of Rho and Ras proteins using fluorescence spectroscopy (15). Besides much lower consumption of proteins and nucleotides as compared to the HPLC-based assay, tamraGTP hydrolysis assay allows to monitor the real-time kinetics of the hydrolysis reaction of the Ras and Rho families.

1. A solution of 0.1 μM tamraGTP (stock solution of >1 mM) is preincubated in a fluorescence cuvette in GAP buffer at a final volume of 600 μL and at 25°C for at least 5 min.
2. Set fluorescence spectrometer at an excitation wavelength of 546 nm and an emission wavelength of 583 nm, with an integration time of at least 2 s and a recording time for each data point of 20 s.
3. Add 1.8 μL from a 50 μM stock solution of the nucleotide-free GTPase (0.15 μM final concentration) to observe complex formation with the nucleotide through a fast and strong increase in fluorescence.
4. After this initial phase of nucleotide association, monitor the significant fluorescence decay as a result of GTP hydrolysis, which lasts between 0.5 and 6 h, depending on the GTPase variant used (15).
5. Continue the measurement until no further decrease in fluorescence can be observed.
6. Evaluate obtained data by single-exponential fitting with scientific software, e.g., the Grafit program, to obtain the observed rate constant (k_{obs}) for the respective concentration of the GAP protein (see Fig. 2b, inset).

3.8.2. Measurement of Fast Reactions

Measure GAP-stimulated tamraGTP hydrolysis by a stopped-flow instrument (see Subheading 3.7.2), but use appropriate settings to detect the tamra fluorescence.

1. Wash the drive syringes of the instrument several times with 5–10 mL of GAP buffer and adjust the temperature to 25°C.

2. Prepare two different samples in GAP buffer at room temperature (25°C) and a final volume of 1,000 µL: (1) one sample contains 0.6 µM nucleotide-free GTPase and 0.4 µM tamraGTP; (2) the other sample contains the GAP protein (at varying concentrations of 0.2–200 µM that depend on the activity of the GAP for the respective GTPase) (see Note 23).
3. Load each sample into one of the two drive syringes of the stopped-flow instrument.
4. Set the excitation wavelength for the tamra nucleotides to 546 nm and detect the fluorescence with a cutoff filter mounted in front of a photomultiplier (570 nm for tamra nucleotides).
5. Start the measurement with the supplied stopped-flow software which initiates the pushing of the two syringes containing the samples to the sample cell. At this stage, both samples join together and rapidly mix to a final volume of about 50–75 µL. Repeat the mixing and fluorescence recording event up to 11 times until all volume in the drive syringe reservoir is consumed.
6. Fit the data single exponentially with, for example, the Grafit program, to provide the hydrolysis rates. GAP proteins of Rho GTPases vary in their activity in stimulating hydrolysis from 10^{1} to 10^{-3} s^{-1} (see Fig. 2b).

3.9. GTPase–Effector Interaction

Fluorescence-based measurement of the interaction between mantGppNHp-bound GTPases and their effectors can be investigated in different ways. A direct method for the time-resolved detection and quantification of interactions is the first step of analysis. However, some GTPase/effector interactions cannot be monitored by direct fluorescence measurements. In these cases, two alternative approaches can be utilized, the guanine nucleotide dissociation inhibition (GDI) assay and equilibrium fluorescence polarization.

3.9.1. Kinetic Measurements (Direct)

To study GTPase–effector interaction, mant-labeled nonhydrolyzable GTP analogs, such as mantGppNHp in complex with the GTPase, can lead to a large change of the fluorescence intensity, like in the case of the Cdc42/WASp interaction (18). In such a kinetic approach, the association and dissociation rates of the effector interaction with mantGppNHp-bound GTPase can be measured using a stopped-flow instrument as described in Subheading 3.7.2.

The association rate constant k_{on} can be measured by using the stopped-flow setup for fast kinetics as follows:

1. Wash the drive syringes of the instrument several times with 5–10 mL effector buffer and adjust the temperature to 25°C.
2. Prepare two different samples in effector buffer at room temperature (~25°C) and a final volume of 1,000 µL: (1) one sample contains 0.2 µM of mantGppNHp-bound GTPase; (2)

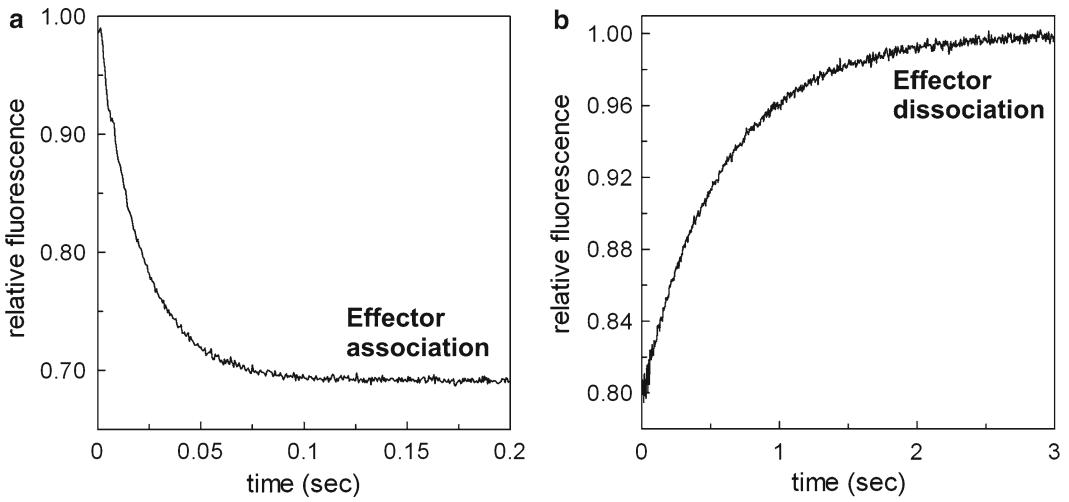

Fig. 3. Association and dissociation reactions for the GTPase–effector interaction. (**a**) A sample containing 0.1 μM Cdc42·mantGppNHp was rapidly mixed with 2 μM WASP in a stopped-flow instrument to monitor the WASP–Cdc42 association in real time. The data was fitted as single-exponential decay to obtain an extremely fast k_{obs} value for association of 48.4 s^{-1}. The association rate constant (k_{on}) can be measured and evaluated by varying the effector concentrations. (**b**) A sample containing 0.1 μM Cdc42·mantGppNHp and 2 μM WASP was rapidly mixed with 10 μM Cdc42·GppNHp in a stopped-flow instrument to monitor the WASP displacement from its complex with Cdc42·mantGppNHp to measure the dissociation rate constant (k_{off}) 1.65 s^{-1}. The ratio of k_{off} divided by k_{on} gives the dissociation constant (K_d) for this bimolecular interaction.

the other sample contains the effector protein (at varying concentrations of 0.2–100 μM depending on the affinity of the effector for the respective GTPase).

3. Next steps are similar to procedures described in Subheading 3.7.2, steps 3–5.
4. Analyze the data by fitting the data for each effector protein concentration with a monoexponential function (see Fig. 3a). Plot the resulting k_{obs} values against the effector concentration and determine the slope of the resulting line, which is k_{on}.

The dissociation rate constant k_{off} can be measured as follows:

1. Mix mantGppNHp-bound GTPase (0.1 μM for high-affinity or 0.5 μM for low-affinity binders in effector buffer) and the effector (0.4 μM for high-affinity or 2 μM for low-affinity binders in effector buffer) with unlabeled GppNHp-bound GTPase (10 μM for low-affinity or 100 μM for high affinity binders) in the stopped-flow instrument to obtain a single-exponential fluorescence change.
2. Next steps are similar to procedures described in Subheading 3.7.2, steps 3–5.
3. Fit the curve single exponentially to obtain the k_{off} value (Fig. 3b). The dissociation constant K_d can now be calculated from the ratio k_{off}/k_{on}.

3.9.2. Equilibrium Measurements Using Fluorescence Titration (Direct)

The investigations described above (see Subheading 3.9.1) can also be carried out using fluorescence equilibrium titration measurements particularly if there is no stopped-flow instrument or if only small amounts of protein are available. In this type of analysis, which has been successfully used for Cdc42/PAK (19), the fluorescence level is measured in dependence of the concentration of the unlabeled reaction partner.

1. Incubate 0.1 μM mantGppNHp-bound GTPase in effector buffer in a quartz cuvette (volume 600 μL) at 25°C in a fluorescence spectrometer and follow fluorescence emission at 450 nm (excitation 366 nm) until the signal is stable.
2. Titrate increasing concentrations of the effector (0.01–100 μM), and wait after each titration step until you reach a stable emission signal level indicating equilibrium.
3. Continue the titration steps until there is no further change of the fluorescence signal indicating that the system is saturated.
4. Plot the fluorescence intensities against the respective effector concentration and fit according to ref. 13 to obtain the equilibrium dissociation constant eK_d.

3.9.3. Measurements of Nucleotide Dissociation Inhibition Through Effector Binding (Indirect)

Some GTPase–effector interactions cannot be monitored by direct fluorescence measurements, but the large fluorescence decay on dissociation of bound mantGppNHp can be utilized to determine indirectly the binding affinity of effector domains for their GTPases as described for Rac/PAK (20, 21), Cdc42/WASp (20), and Rho/Rhotekin, Rho/PKN, and Rho/Rho kinase interactions (22). This method is based on the observation that guanine nucleotide dissociation from the GTPase is inhibited by interaction with effectors (GDI effect), as the effectors predominantly bind close to the nucleotide-binding region of the GTPase. According to the used effector concentration, a fraction of the GTPase is bound by the effector, thus slowing down the nucleotide dissociation. The observed nucleotide dissociation rate is a combination of the dissociation rates of free and effector-bound GTPase.

1. Add 0.2 μM mantGppNHp-bound GTPase together with different concentrations of the full length effector or the GTPase-binding domain (0.2–2.0 μM for high-affinity interactions and up to 100 μM for low-affinity binders) into a quartz cuvette containing effector buffer, equilibrate at 25°C, and monitor the fluorescence emission at 450 nm (excitation at 366 nm).
2. Add 20 μM unlabeled GppNHp into the solution and mix to start the reaction. For every experimental setup, four reactions can be measured simultaneously if the fluorescence spectrometer is equipped with a four-position cuvette holder.

3. For better comparability of the different setups, the intrinsic nucleotide dissociation in the absence of the effector should be determined in one of the four reactions.
4. Evaluate obtained data by single-exponential fitting with scientific software, e.g., the Grafit program, to obtain the equilibrium dissociation constant (eK_d) as a consequence of the GDI effect.

3.9.4. Equilibrium Measurements Using Fluorescence Polarization Titration (Direct)

Fluorescence polarization can also be used to determine the equilibrium dissociation constant (eK_d), and works best if the effector protein is larger than 40 kDa. In cases where the effector proteins are to small, like in the isolated GTPase-binding domains, a GST- or maltose-binding protein fusion of the effector can be used to obtain sufficiently large polarization signal changes (23, 24).

1. Incubate 0.2 μM mantGppNHp-bound GTPase in effector buffer (see reagents and buffers) in a fluorescence cuvette (volume 600 μL) at 25°C and monitor the fluorescence polarization in a fluorescence spectrometer (emission at 450 nm, excitation 366 nm) until the signal is stable.
2. Titrate increasing concentrations of the effector (0.01–100 μM), and wait after each titration step until a stable polarization signal level is reached, indicating equilibrium.
3. Continue the titration steps until there is no further change of the polarization signal, indicating that the system is saturated.
4. Fit the concentration-dependent binding curve using a quadratic ligand-binding equation (24) to obtain the equilibrium dissociation constant (eK_d) for the respective GTPase–effector interaction.

3.10. Anticipated Results

The elucidation of the molecular switch mechanism of the GTPases and particularly their specificities and affinities for regulators and effectors requires the dissection of such interactions at the molecular level by utilizing sensitive biochemical assays. Fluorescence spectroscopic methods provide researchers with a number of tools for studying the intercommunication of a GTPase with nucleotides and binding partners. Compared to qualitative assays (e.g., the filter-binding assay or thin-layer chromatography, which contain between 3 and 6 data points), the fluorescence methods described in this chapter allow to monitor the activity of GEFs and GAPs in real time as well as the interaction with effectors at which every single measurement consists of at least 400 data points per reaction trace. These assays are highly sensitive and, in principle, reproducible provided that the proteins and reagents are carefully prepared from high-purity materials and tested for quality. Thus, optimal gene expression and protein purification as well as the quality of fluorescent nucleotide-bound GTPases and other components,

including GEFs, GAPs, effector proteins, and nucleotide derivatives, are prerequisites for reliable and reproducible measurements. In all assays described in this chapter, a decrease in fluorescence should mostly be monitored in a time-dependent manner; in other cases, an increase in fluorescence may be observed. However, another important aspect to be considered is the fluorescence offset, which represents the final fluorescence. This should be relatively similar for all experiments (1) under the same concentrations of the fluorescent nucleotides in the complex with the GTPase and (2) independent of the GEF, GAP, and effector concentrations.

4. Notes

1. Remember to add the required antibiotics to the TB medium to maintain transformed plasmids. In case of using BL21 (DE3) Codon plus RIL, BL21 (DE3) pLysS, or Rosetta (DE3) strains, chloramphenicol (25 mg/L) needs to be added.
2. Cultures are carried out usually in 2.5–20-L scale (depending on the expression level and yield of the particular protein).
3. The small GTPases as well as GEF and GAP proteins, including their catalytic domains, are usually expressed at an OD_{600} of 0.6–0.8 with 0.1 mM IPTG and at 18–25°C overnight.
4. This step is carried out in order to remove residual medium.
5. Cryo preservation through storage at −20°C also helps to improve the efficiency of bacterial lysis.
6. Optionally, the wave duty cycle function of the ultrasonic instrument can be used to reduce heat production and free radical formation.
7. A color change from very milky to slightly more translucent should be observed and can be used as an indicator for cell disruption.
8. This method permits cell disruption in smaller samples (≥200 μL and ≤200 mL).
9. This step removes all traces of alcohol in which the instrument usually is stored to prevent microbial growth.
10. The microfluidizer system provides a convenient and efficient method for cell lysis of larger cell suspensions (≥5 mL to several liters).
11. When purifying guanine nucleotide-binding proteins, it is mandatory to add 0.1 mM GDP during the first affinity chromatography on GSH sepharose and magnesium ions to the standard buffer, which are essential especially in the case of low-affinity GDP/GTP-binding proteins. Always determine the

GDP concentration by HPLC in addition to determining protein concentration.

12. ATP is used for removing chaperons which might be associated with the desired protein and the high concentration of KCl to remove proteins which unspecifically bind to the column or to the GST fusion protein of interest. Note that the absorbance at 280 nm is not reaching the previous baseline level due to the absorbance of ATP present in the buffer applied.
13. Be aware that one has to readjust the pH of the elution buffer with NaOH due to the acidity of GSH.
14. The size and, therefore, the choice of the column size depend on the amount of purified protein and its molecular mass: 16/60 for protein amounts of ≤30 mg or 26/60 for 30–100 mg. If the protein amount to be purified exceeds 100 mg, divide the sample into several portions of ≤100 mg and perform several consecutive runs.
15. After cleaving the protein with protease, the GST tag is not attached to the desired protein anymore. Therefore, while passing again on a GSH sepharose column, the desired protein passes without binding or retention through the column and elute as flow through.
16. Freezing and thawing of protein solutions is a very critical step and has a large impact on protein stability. It is absolutely mandatory to freeze a protein solution in liquid nitrogen and to store it afterward at –20°C or even –80°C. For longer storage periods, the latter is recommended. Before freezing a protein, a small-scale test whether the protein can be frozen in plain standard buffer is recommended, and addition of supplements like glycerol or sucrose might help to prevent protein denaturation during freezing. Thawing of protein solutions should be fast by warming up the vials in the hands. For each protein, the optimal strategy has to be elaborated.
17. Residual alkaline phosphatase-coupled beads might interfere with subsequent spectrofluorometric assays performed with the nucleotide-free GTPase.
18. Releasing GDP (or GTP in the case of the constitutive mutants) from the GTPase and reloading with fluorescent nucleotides, as described above, are prerequisites to perform fluorescence measurements. The incubation time for preparing the nucleotide-free proteins varies among GDP/GTP-binding proteins and has to be established for every GTPase. GppNHp is resistant to phosphodiesterase and thus should not be used in place of Gpp(CH_2)p.
19. In order to obtain reliable and reproducible kinetic data, the protein and nucleotide quality needs to be high. Thus, nucleotides

with more than 90% purity should be used and, if necessary, additional purification steps should be carried out to obtain nucleotides and proteins of high purity.

20. Spectroscopic measurements like the fluorescence assays described in this chapter require the use of very clean quartz cuvettes, filtered and degassed buffers, as well as protein solutions without precipitate or any other solid material. Otherwise, light dispersion will take place and the signal-to-noise ratio will be poor. It is, therefore, very important to centrifuge the protein solution immediately before using.
21. Because the samples are mixed 1:1, all stock solutions for components of the samples should be 2× and are described in this section of the protocol as 2× concentrated.
22. Example: Dilute 2 µL of a 100 µM mantGDP-bound GTPase solution in 998 µL of GEF buffer to obtain a 0.2 µM solution of the respective mantGDP-bound protein. A tenfold excess of the GEF protein usually is the first choice to determine the activity of the GEF protein. For that, mix 20 µL of a 100 µM GEF solution (20 µM final concentration) and 4 µL of a 10 mM GDP solution (40 µM final concentration) in 976 µL of GEF buffer. Accordingly, both solutions have a final volume of 1 mL.
23. Example: Dilute 12 µL from a 50 µM solution of nucleotide-free GTPase (0.6 µM final concentration) in 968 µL of GAP buffer and add 20 µL of a 20 µM tamraGTP solution (0.2 µM final concentration) just prior to loading the drive syringes of the stopped flow with your sample. In a double mixing stopped-flow system, both components (tamraGTP and GTPase) can be premixed for a defined short time before the GAP reaction is started.
24. The amounts of proteins and (fluorescent) nucleotides required are rather dependent on the assay used. For the determination of intrinsic nucleotide dissociation or intrinsic GTP hydrolysis in a cuvette (at a final volume of 600 µL), between about 10 and 20 µg nucleotide-bound GTPase is required for three identical experiments. At least 60 µg of catalytic domains of GEF or GAP proteins (with 250–300 amino acid residues) is needed for one experiment to measure the specificity and activity of these regulatory proteins (19). A stopped-flow experiment requires 3–6 µg of GTPase and 15–200 µg of GEFs, GAPs, or effectors but provides an averaged value obtained from 5 to 7 identical measurements. However, between 2 and 10 mg of binding proteins are required to quantitatively analyze the GEF or GAP activities or the effector-binding affinity.

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Short Communication

New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm

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Abstract

Major advances have been made in understanding the structure, function and regulation of the small GTP-binding proteins of the Rho family and their involvement in multiple cellular process and disorders. However, intrinsic nucleotide exchange and hydrolysis reactions, which are known to be fundamental to Rho family proteins, have been partially investigated in the case of RhoA, Rac1 and Cdc42, but for others not at all. Here we present a comprehensive and quantitative analysis of the molecular switch functions of 15 members of the Rho family that enabled proposing a GTP-bound state for the rather uncharacterized isoforms RhoD and Rif under equilibrium and quiescent conditions.

Keywords: Rho family, nucleotide dissociation, nucleotide association, GTP hydrolysis, Rif, RhoD.

Members of the GTP binding proteins of the Rho family have emerged as key regulatory molecules that couple changes in the extracellular environment to intracellular signal transduction pathways. So far, 22 human members of the Rho family have been identified (Wennerberg and Der, 2004) with the best-characterized members being Rac1, RhoA, and Cdc42 (Etienne-Manneville and Hall, 2002). They act as intracellular molecular switches by cycling between active (GTP-bound) and inactive (GDP-bound) states (Dvorsky and Ahmadian, 2004). Activation of Rho proteins results in their association with effector molecules that subsequently activate a wide variety of downstream signaling cascades (Bishop and Hall, 2000; Burridge and Wennerberg, 2004) therefore regulating many important physiological and pathophysiological processes in all eukaryotic cells (Heasman and Ridley, 2008).

Rho family proteins share, like any other members of the Ras superfamily, a core GTP-binding (G) domain with five conserved sequence motifs (G1-G5; Supplementary Figures S1 and S2). These motifs are involved in nucleotide binding and hydrolysis (Wittinghofer and Vetter, 2011). The cycle between inactive and active states (Dvorsky and Ahmadian, 2004) engage at least two regions of the protein, switch I (G2) and Switch II (G3), which undergo structural rearrangements and transmit the ‘off′ to ‘on′ signal to downstream effectors. The cycle is driven by two rather slow reactions, the GDP/GTP exchange and the GTP hydrolysis, which are accelerated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Dvorsky and Ahmadian, 2004).

In resting cells, Rho proteins are believed to persist in their inactive GDP bound forms alone or alternatively in complex with the GDIs (Dovas and Couchman, 2005). This scenario is based on the assumption that the intrinsic GTP hydrolysis reaction of a GTP-binding protein is faster than the intrinsic GDP/GTP exchange reaction resulting in an inactive GDP-bound protein. As experimental data addressing the intrinsic properties of several members of the Rho family using different methods and conditions (Fiegen *et al.*, 2004; Haeusler *et al.*, 2003; Jaiswal *et al.*, 2011; Zhang *et al.*, 2000) is rather fragmentary, we set out to analyze sequence-structure-function relationships of all fifteen members of the Rho family, including RhoA, RhoB, RhoC, Rnd1, Rnd2, Rnd3, Rac1, Rac2, Rac3, RhoG, Cdc42, TC10, TCL, RhoD and Rif, regarding their intrinsic properties, including nucleotide binding and GTP hydrolysis.

Taking advantage of the large change in fluorescence intensity during the time course of the association of the nucleotide-free protein with fluorescent mantGDP, which can be monitored after rapid mixing of two components in a stopped-flow instrument (Jaiswal *et al.*, 2011), we measured association kinetics of mantGDP with 12 nucleotide-free Rho-related proteins (except for the Rnd proteins, see below). As shown in Supplementary Fig. S3A, the association of 0.1 μM mantGDP with increasing concentrations of nucleotide-free RhoA (0.05–2.0 μM) led to the incremental increase in fluorescence intensity. Observed rate constants (k_{obs}) plotted against the concentration of nucleotide-free Rho proteins resulted in the determination of the association rate constants (k_{ass}) for the analyzed Rho proteins (Table 1, Supplementary Fig. S3B). Direct comparison of mantGDP association of twelve Rho-related proteins revealed that RhoA, Rac1, Rac3 and Cdc42 have strikingly rapid nucleotide association compared especially to that of RhoB, Rac2 and TC10, which are more than 100-fold slower.

To complement the nucleotide binding properties, we next measured the GDP dissociation of Rho proteins. We mixed mantGDP-bound proteins with excess amounts of non-labeled GDP and measured the fluorescence decay followed over time. As shown in Supplementary Figure S4, mantGDP dissociation from Rho proteins lasts up to 36 h to be completed corresponding to an extremely low dissociation rate constants (k_{diss}) (Table 1). These data clearly demonstrate a strict dependence of Rho protein signaling on their activation by GEFs, which are required to speed up to such extremely slow reaction rates. Circumstances turned out to be different for RhoD and Rif. Dissociation of mantGDP from these proteins was relatively fast exceeding k_{diss} values of other Rho proteins by almost three orders of magnitude (Table 1, Supplementary Figure S4). Interestingly, the nucleotide binding properties of RhoD and Rif are rather comparable to that of Rac1b, an alternative splice variant of Rac1 (Fiegen *et al.*, 2004; Haeusler *et al.*, 2006). Rac1b, due to a 19-amino acid insertion next to the switch II region (Fiegen *et al.*, 2004), revealed a 40-fold increase in nucleotide dissociation (Table 1).

Taken together, dissociation constants (K_d) calculated as *ratios*, k_{diss}/k_{ass} revealed that the affinities of RhoA, Rac1 and Cdc42 proteins for mantGDP are in the high picomolar range, which was 1000-fold lower in the nanomolar range in the case of RhoD and Rif (Table 1). In contrast to this data, nucleotide binding affinity of RhoA, Rac1 and Cdc42 has been previously reported to be at a submicromolar, 20 000-fold lower range (Zhang *et al.*, 2000). This discrepancy is due to the different methods used for investigating the nucleotide association. Unlike the single time point (6 h) filter binding assay (Zhang *et al.*, 2000), our

study is based on individual real-time fluorescence measurements as shown in Supplementary Figures S3 and S4. In addition, our data on high affinity nucleotide binding for most Rho family proteins are in the same range as reported before for the members of other families such as Ras, Ran, Rab and Arf (John *et al.*, 1990; Kabcenell *et al.*, 1990; Klebe *et al.*, 1995; Randazzo *et al.*, 1995; Simon *et al.*, 1996).

The GTPase reaction, which terminates downstream signaling, is a hallmark of the molecular switch function of most Rho family proteins (Li and Zhang, 2004; Rittinger *et al.*, 1997; Scheffzek *et al.*, 1998). Intrinsic GTP hydrolysis of GTP-binding proteins is a second crucial function that we characterized for different Rho proteins by monitoring gradual decrease of GTP and the corresponding increase of GDP in the course of GTPase reaction. The findings obtained in this study were evaluated by single exponential fitting (Supplementary Figure S5) and calculated rate constants for the intrinsic GTP hydrolysis (k_{cat}) are summarized in the Table 1. While Rho proteins exhibit marked differences in their guanine nucleotide binding properties, the rates of GTP hydrolysis appeared to be rather similar. Interestingly, there are up to 5-fold biochemical differences for RhoD and Rif that belong to moderately hydrolyzing members of the Rho family. Rac1b exhibits the slowest intrinsic GTPase reaction amongst the Rho family proteins with a rate of 5.8×10^{-5} per s (Table 1) (Fiegen *et al.*, 2004).

A comparison of the rate constants for the GDP dissociation (k_{diss} values) and the GTP hydrolysis (k_{cat}) indicates that RhoD and Rif exhibit, similarly to Rac1b, strikingly a faster nucleotide exchange than GTP hydrolysis (Table 1; Figure 1). This result is unexpected given that k_{cat} is conventionally higher relative to k_{diss} so that the majority of the Rho family proteins under resting conditions exist predominantly in the inactive, GDP-bound form at a steady state (Figure 2A). We thus propose that RhoD and Rif, unlike the conventional members of the Rho family, resemble Rac1b (Fiegen *et al.*, 2004) by persisting mainly in the active state under resting conditions (Figure 2B).

Rnd1, Rnd2, Rnd3 and RhoH/TTF represent a completely distinct group of proteins within the Rho family. In addition to other deviations, they do not share several conserved and essential catalytic amino acids, including G12 and Q61 (Rac1 numbering; Supplementary Figure S1). Thus, they can be considered as GTPase-deficient, Rho-related GTP binding proteins (Fiegen *et al.*, 2002; Garavini *et al.*, 2002; Gu *et al.*, 2005; Li *et al.*, 2002). The first indication for a loss of a functional GTPase machinery was the finding that purified Rnd proteins from bacteria were only obtained in the GTP-bound state similarly to the purified

constitutive active forms of Ras and Rho (data not shown). Nonetheless, we were interested in analyzing the residual GTPase activity of these proteins and set out to incubate the purified GTP-bound proteins at 25°C and to perform HPLC measurements, which were aborted at the fourth day due to protein precipitation. The Rnd proteins exhibited an extremely low GTP hydrolysis activity and contained after four days still 60% GTP, which makes a difference to the conventional members of the Rho family of more than 1000-fold (data not shown). This could not be shown for RhoH/TTF because this isoform could not be isolated as a stable protein. In addition, efforts to prepare nucleotide-free Rnd proteins failed so we could not measure k_{ass} values for this group of proteins. Incubation of GTP-bound Rnd1 and Rnd3 in the presence of fluorescent tamraGTP (Eberth *et al.*, 2005) showed that these proteins are, in principle, able to undergo nucleotide exchange with dissociation rate constants rather comparable to RhoA (data not shown). These data strongly suggest that Rnd proteins are not regulated by a conventional GDP/GTP cycling mechanism and due to a high k_{diss}/k_{cat} ratio accumulate also in GTP bound form in cells (Figure 2A and C) (Fiegen *et al.*, 2002). The activity of these atypical members of the Rho family appears to be regulated by posttranslational modification, including phosphorylation (Riou *et al.*, 2010), rather than by the function of GEFs and GAPs.

Taken together, the majority of the Rho family proteins, although very inefficient GTP hydrolyzing enzymes, rests in the inactive state in quiescent cells because the GTP hydrolysis is in average two orders of magnitude faster that the intrinsic GDP/GTP exchange. It is generally accepted that such a paradigm for two-state molecular switches depends on the upstream signals so that its equilibrium would be actively shifted to the GTP-bound state. However, this study clearly showed that such a paradigm is not universal if we look at the unique features of RhoD and Rif regarding their `shifted´ ratio k_{diss}/k_{cat} (Figure 1; Table 1). This implies that these two members of the Rho family may not be necessarily dependent on GEFs especially in slow cellular processes but rather on GAPs in order to switch off their signal transduction.

Important questions about both the cause and regulatory implications of RhoD and Rif are raised by observed dramatic differences. We first focused on structural-functional features of RhoD and Rif proteins and analyzed their amino acid sequences in two ways. On the basis of phylogenetic tree analysis, the relationships among Rho family proteins were investigated. As shown in Supplementary Figures S1B and S2B, RhoD and Rif emerged as evolutionary distinct branches from Rac-, Cdc42 and Rho subfamily regardless of considering all amino

acids or only the guanine nucleotide binding residues for phylogenetic analysis. RhoD and Rif were also demonstrated as distinct evolutionary new branch, which emerged very late in evolution (Boureux A *et al.*, 2007). These findings support distinct intrinsic properties of RhoD and Rif revealed by our experimental data.

A detailed evaluation of the primary sequences of Rho family proteins using a multiple sequence alignment showed that a large number of conserved amino acids are different throughout the G-domain of RhoD and Rif (Supplementary Figure S1). Interestingly, there is a limited number of moderate deviations in three out of five consensus nucleotide binding motifs (G1-G5), which are common characteristics of the Ras superfamily (Dvorsky and Ahmadian, 2004). To obtain additional insights into the impact of the unique features of RhoD and Rif, we used various available structures of the Rho proteins (Supplementary Table S2) and compared them on the basis of a multiple sequence alignment (Supplementary Figure S1). It turned out that the majority of deviating amino acids in G1, G2 (also called switch I) and G4 motifs of RhoD and Rif are not involved in nucleotide binding and/or hydrolysis. Variations in a close vicinity of nucleotide reside in four residues (RhoA numbering: A15G, C20S, V/E33S/H, V/I35T/A; Figure 2D). Wrch1, another member of the Rho family, has been reported to be a fast cycling protein due to a rather high intrinsic mantGDP dissociation rate of 0.012 s^{-1} (Shutes *et al.*, 2006). This protein, similarly to RhoD and Rif, also contains a serine at the corresponding position of the conventional cysteine (Supplementary Figure S1). Sondek and coworkers have shown that the substitution of Cys18 and Val33 of Cdc42 (Cys20 and Val35 in RhoA) with alanines results in a 17- and 6-fold increase in the mantGDP dissociation, respectively (Rossman *et al.*, 2002). Of particular note, the C20A mutant leads to a fast cycling phenotype for Cdc42 and produces a dominant negative form of Cdc42 capable of inhibiting RhoGEFs both *in vitro* and *in vivo*.

It is important to emphasize the role of amino acids that are distant and ‘on a back side’ of the nucleotide binding site but may significantly influence the overall dynamics of the proteins and thus also their intrinsic functions (Haeusler *et al.*, 2003). Excluding homologous residues, there are five and eleven amino acid variations in the G domain of Rac2 and RhoB, respectively, as compared to Rac1/Rac3 and RhoA/RhoB (Supplementary Figure S1). These residues are mostly located at the surface and are neither in the vicinity of switching regions nor at the nucleotide binding site (data not shown). Varying properties observed for these proteins cannot be therefore explained by the direct influence of varying amino acid but rather by changes in the overall structural flexibility. Mutational analysis of TC10 has shown that

replacement of several residues, including Glu110 (Figure S1), is required to revert aberrant TC10 properties to that of Cdc42 (Dvorsky *et al.*, unpublished). Association rate constants of RhoB, Rac2 and TC10 are relatively low (Table 1), but their very low k_{diss} and k_{cat} values clearly indicate that these proteins, like their Rho, Rac and Cdc42 homologues, are regulated by GEFs and GAPs. This notion is supported by kinetic analysis in the presence of different specific Dbl proteins and RhoGAPs (Jaiswal *et al.*, unpublished). We hypothesize that the kinetic changes are the consequence of the amino acid deviations at the surface of RhoB, Rac2 and TC10, which may generate new specific binding sites and abrogate interactions of otherwise common binding partners. An example for the latter case is RhoGDI, which has been reported not to interact with RhoB and TC10 (Michaelson *et al.*, 2001).

Rif contains a serine at position 51, which is normally an almost invariant threonine (T35 in Rac1 or Cdc42 and T37 in RhoA; Figure 2D and Supplementary Figure S1). The function of this threonine in GTP-binding proteins, which is GTP-dependent, has been described to be the stabilization of the switch function for the interaction with the downstream effectors (Spoerner *et al.*, 2001). Accordingly, this is achieved through its contacts with the γ-phosphate and the magnesium ion upon the GDP/GTP exchange (Dvorsky and Ahmadian, 2004; Ihara *et al.*, 1998; Wei *et al.*, 1997). This suggests that the threonine to serine deviation at position 51 in Rif is not a reason for the very high k_{diss} value for GDP. This deviation may rather affect Rif signaling to its effectors as it has been established for Ras (Joneson *et al.*, 1996; White *et al.*, 1995). One of the most prominent deviations outside the nucleotide binding motifs, which may indeed contribute to the fast GDP dissociation from RhoD and Rif, is the substitution of an invariant proline (P106 in Rac1 or Cdc42 and P108 in RhoA; Fig. 2D) for K120 in RhoD and R122 in Rif (Figure S1). The presence of a proline appears to be critical for the structural integrity of both α3 helix, which in turn stabilizes the G1 and G2 motifs. Taken together, these effects may contribute to a decrease in the GTPase reaction as observed in this study (Table 1).

In conclusion, this study has provided, for the first time, firm evidence for critical differences in the intrinsic biochemical properties not only among the highly related Rho and Rac isoforms but also among all members of Rho family beyond RhoA, Rac1 and Cdc42. The most striking findings represent the special features of RhoD and Rif that might ultimately not follow the conventional switch mechanism – supporting shifted paradigm of classical regulation of small GTP binding proteins. This study is thus a good starting point for the verification of such hypothesis by conducting more detailed biochemical analysis of Rho

family proteins in the interplay with 3 GDIs, 75 GEFs and 85 GAPs, and their interaction with and activation of more than 100 effector proteins.

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Tables and figures

Table 1 Nucleotide-binding and hydrolysis properties of the Rho proteins.

Rho[a] proteins	GDP binding[b]			GTPase[b]	k_{diss}/y_{cat}
	k_{ass} ($\mu M^{-1}*s^{-1}$)	k_{diss} (s^{-1})	$K_d = k_{diss}/k_{ass}$ (nM)	k_{cat} (s^{-1})	
RhoA	6.2496	0.000024	0.004	0.00060	0.040
RhoB	**0.0464**	0.000049	**1.056**	0.00042	0.117
RhoC	0.2095	0.000044	0.210	0.00068	0.065
Rac1	4.1487	0.000045	0.011	0.00160	0.028
Rac2	**0.0536**	0.000074	**1.380**	0.00144	0.051
Rac3	2.8303	0.000069	0.024	0.00175	0.039
RhoG	0.5732	0.000019	0.033	0.00204	0.009
Cdc42	2.4620	0.000042	0.017	0.00184	0.023
TC10	**0.0535**	0.000048	**0.897**	0.00030	0.160
TCL	0.1556	0.000100	0.643	0.00064	0.156
RhoD	0.7675	**0.013600**	**17.719**	**0.00059**	**23.051**
Rif	0.2549	**0.003900**	**15.300**	**0.00064**	**6.094**
Rac1b[c]	1.1000	**0.001800**	**1.636**	**0.000058**	**31.035**

[a]Rho proteins, including human RhoA (aa 1-181), human RhoB (aa 1-181), human RhoC (aa 1-181), human Cdc42 (aa 1-178), human Rac1 (aa 1-184), human Rac2 (aa 1-192), human Rac3 (aa 1-192), human TC10 (aa 2-193), human TCL (aa 2-197), murine RhoD (aa 2-193), human Rif (aa 1-195), human RhoG (aa 1-178), were amplified by standard PCR and cloned in pGEX-4T1 vector and confirmed by DNA sequencing. All proteins were produced as described (Eberth and Ahmadian, 2009). Briefly, the proteins were produced as glutathione transferase (GST) fusion proteins in *Escherichia coli* BL21 (DE3) or BL21(DE3) pLysS or Rosetta. GSH–Sepharose (Pharmacia) was used as the first purification step. After protease cleavage of the GST tags, the proteins were applied to a gel-filtration column (Superdex 75, Pharmacia) and a subsequent GSH–Sepharose column as the final step to obtain a purity of at least 95%. Nucleotide-free proteins were prepared using enzymatic activity of alkaline phosphatase (Roche) and phosphodiesterase (Sigma Aldrich) at 4°C as described (Jaiswal *et al.*, 2012). Fuorescent methylanthraniloyl (mant)-GDP-bound Rho proteins were prepared, and quality were determined as described (Jaiswal *et al.*, 2012). Purified proteins were snap frozen in liquid nitrogen and stored at –80°C.

[b]Nucleotide-binding and hydrolysis was measured as described (Hemsath *et al.*, 2005).

[c]Rac1b data were taken from Haeusler *et al.*, 2003 and 2006. Protein stability was analyzed by keeping them at room temperature for several hours and by freezing (in liquid nitrogen) and thawing them twice. Additional procedures are described in the supplementary information. Bold values indicate significant differences of the observed rate constants and calculated dissociation constants (K_d).

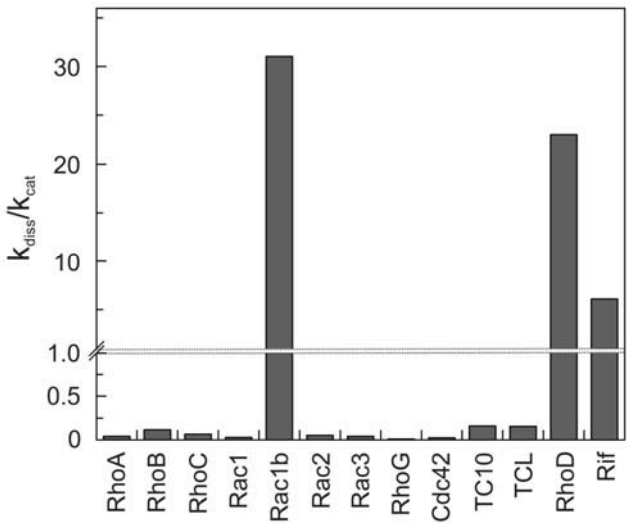

Figure 1 Differential state of Rho family proteins.

The k_{diss}/k_{cat} values (Table 1) are used to emphasize the faster nucleotide exchange versus the GTPase reaction of Rac1b, RhoD and Rif. These proteins, in contrast to conventional members of the Rho family, most likely persist in the active, GTP-bound state under unstimulated resting conditions as characterized by the ratio $k_{diss}/k_{cat} > 1$.

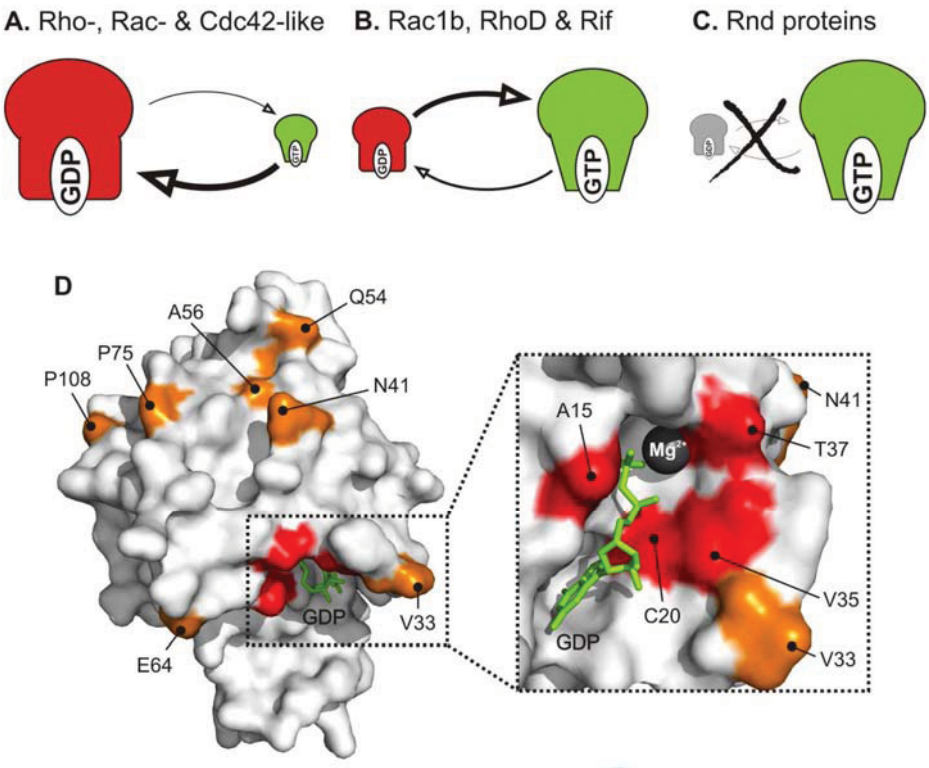

Figure 2 Differential intrinsic functions and mechanisms of Rho proteins.

(A) Conformational changes driven by an extremely slow nucleotide exchange and a relatively fast GTP hydrolysis are an attribute of canonical molecular switches keeping them in their GDP-bound, inactive state under resting conditions. The thickness of the arrows represents different magnitudes of the nucleotide exchange and hydrolysis reaction rates. (B) Alternative molecular switches end up in the GTP-bound, active state due to a faster nucleotide exchange and a slower GTP hydrolysis rates. (C) Lack of a GTPase activity cancels a molecular switch mechanism (highlighted by x), yielding a persistent GTP-bound state. (D) Different intrinsic properties of RhoD and Rif are conceivable only due to amino acids deviating within (red) and outside (orange) of the nucleotide binding site. The crystal structure of RhoA (Protein Data Bank code: 1FTN) in the complex with GDP (green) and Mg^{2+} (black sphere) highlights the most critical residues (RhoA numbering).

Jaiswal *et al.*, Supplementary information

Supplementary Table S1: Rho family proteins in the human genome

No.	Rho proteins	Synonyms	Acc. no.	Aa no.
1	**RhoA**	Rho12	P61586	193
2	**RhoB**	RhoH6	P62745	196
3	**RhoC**	RhoH9	P08134	193
4	**Rac1**	TC-25, p21-Rac1	ACE87731	192
5	**Rac1b**		CAA10732	211
6	**Rac2**	EN-7, Gx, HSPC022	AAM21112	192
7	**Rac3**	p21-Rac3	P60763	192
8	**RhoG**	ARHG	NP_001656	191
9	**Cdc42**	Cdc42Hs	NP_426359	191
10	G25K		P60953	191
11	**TC10**	RhoQ; TC10A	P17081	205
12	**TCL**	RhoJ; TC10B	Q9H4E5	214
13	**RhoD**	RhoM	O00212	219
14	**Rif**	RhoF	Q9HBH0	211
15	Chp1	RhoV; Wrch2; Chp	Q96L33	236
16	Wrch1	RhoU; hG28K	Q7L0Q8	258
17	TTF	RhoH	Q15669	191
18	**Rnd1**	RhoS; RHO6	Q92730	232
19	**Rnd2**	RhoN; RHO7	P52198	227
20	**Rnd3**	RhoE; RHO8; MemB	P61587	244

Rho proteins are presented with their alias names, accession number and number of amino acids. Rho proteins highlighted in bold are investigated in this study.

Supplementary Table 2: Published three-dimensional structure of Rho proteins

No.	Protein	PDB ID	Resolution (Å)	Reference
1	RhoA ^{F25N}	1FTN	2.10	(Wei *et al.*, 1997)
2	RhoA	1A2B	2.40	(Ihara *et al.*, 1998) RhoA
3		1DPF	2.00	(Shimizu *et al.*, 2000) RhoA
4	$^{Q63L, F25N}$	1KMQ	1.55	(Longenecker *et al.*, 2003)
5	RhoB	2FV8	1.90	
6	RhoC	2GCN	1.85	(Dias and Cerione, 2007)
7	RhoC	2GCO	1.40	(Dias and Cerione, 2007)
8	RhoC	2GCP	2.15	(Dias and Cerione, 2007)
9	Cdc42Hs	1AN0	2.80	
10	Cdc42 ^{G12V}	1A4R	2.50	(Rudolph *et al.*, 1999)
11	Cdc42Hs	1AJE	NMR	(Feltham *et al.*, 1997)
12	Cdc42 ^{T35A}	2KB0	NMR	
13	Cdc42Hs ^{F28L}	2ASE	NMR	(Adams and Oswald, 2006)
14	Rac1	2P2L	1.90	(Prehna and Stebbins, 2007)
15	Rac1b	1RYH	1.75	(Fiegen *et al.*, 2004)
16	Rac1b	1RYF	1.75	(Fiegen *et al.*, 2004)
17	Rac2 ^{G12V}	2W2T	1.95	(Bunney *et al.*, 2009)
18	Rac2 ^{G12V}	2W2V	2.00	(Bunney *et al.*, 2009)
19	Rac3	2IC5	1.90	
20	Rac3	2G0N	1.90	
21	Rac3	2C2H	1.85	
22	RhoD	2J1L	2.50	
23	TC10	2ATX	2.65	(Hemsath *et al.*, 2005)
24	Wrch1	2Q3H	1.73	
25	Rnd1	2CLS	2.31	
26	Rnd3	1M7B	2.00	(Fiegen *et al.*, 2004)
27	Rnd3	1GWN	2.10	(Garavini *et al.*, 2002)

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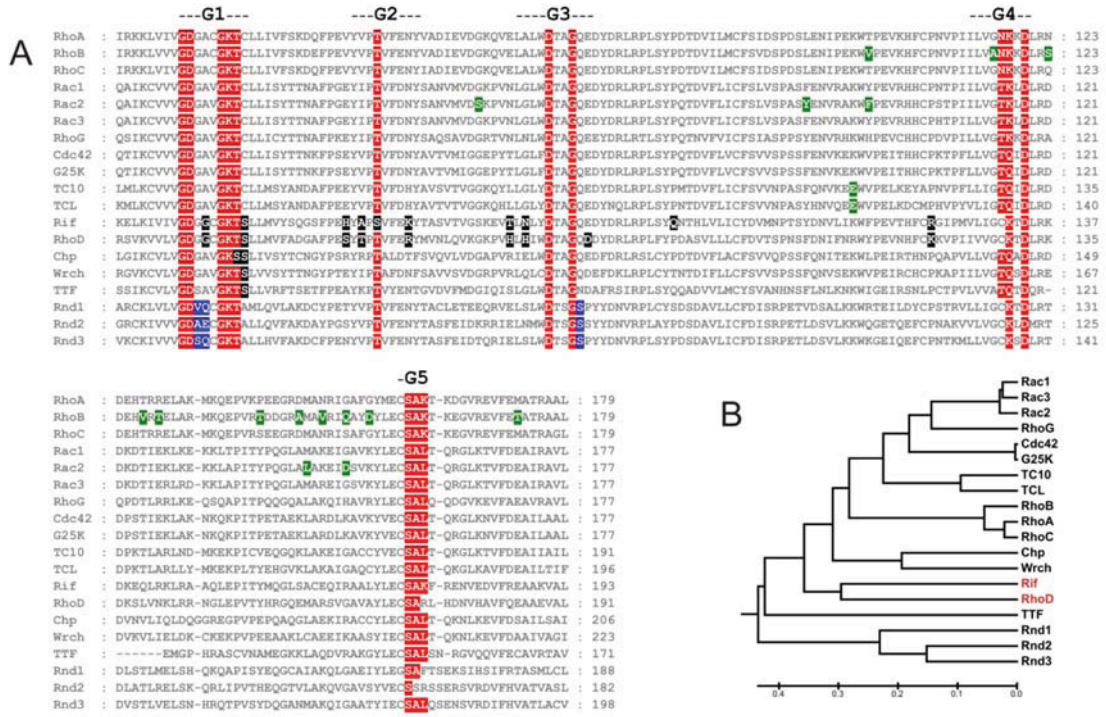
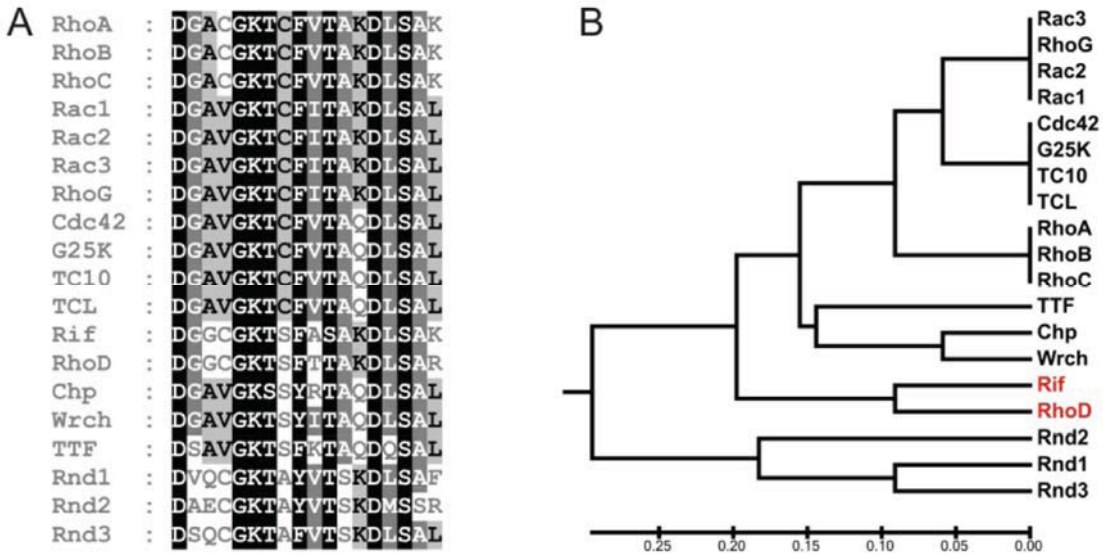
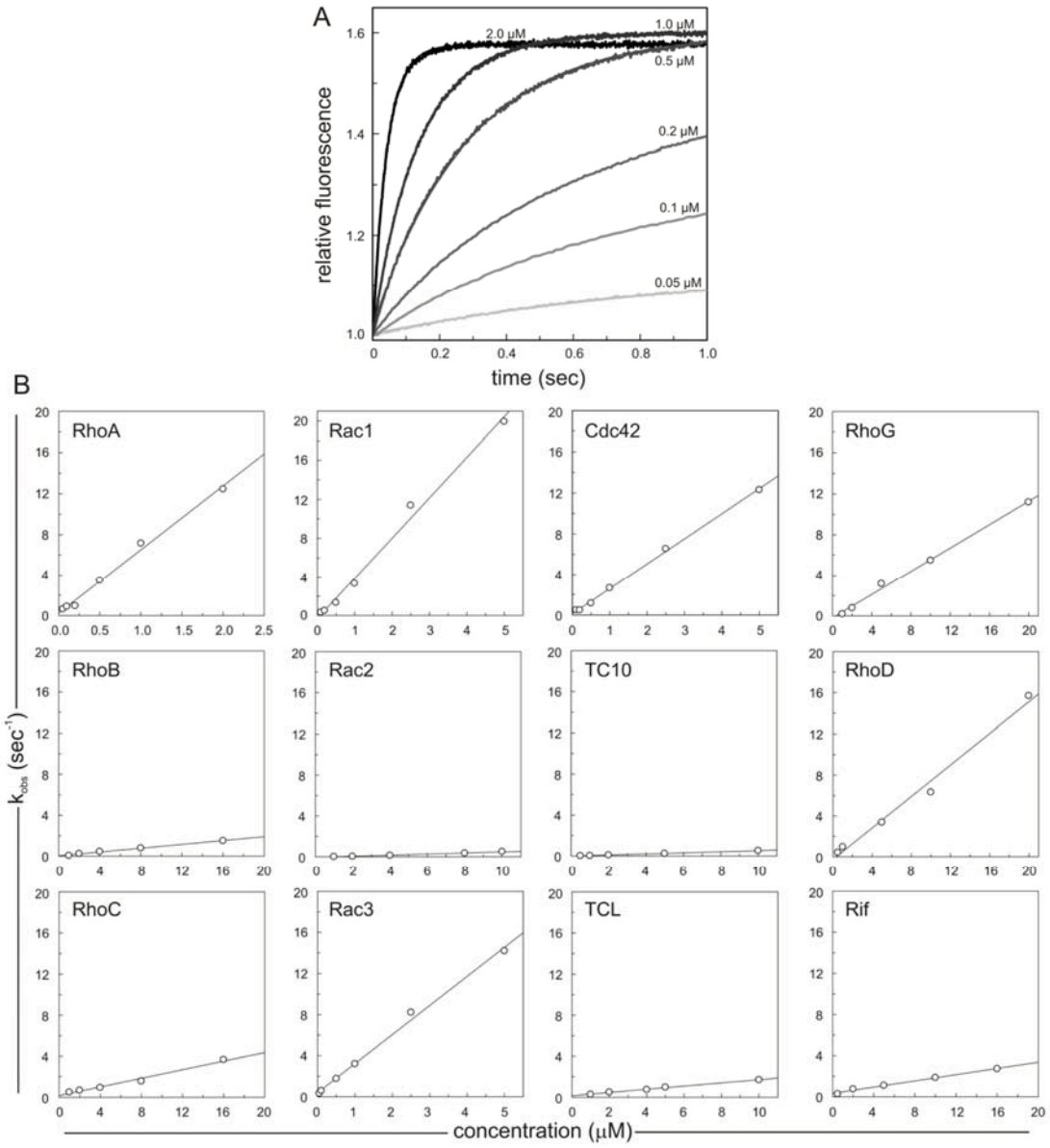


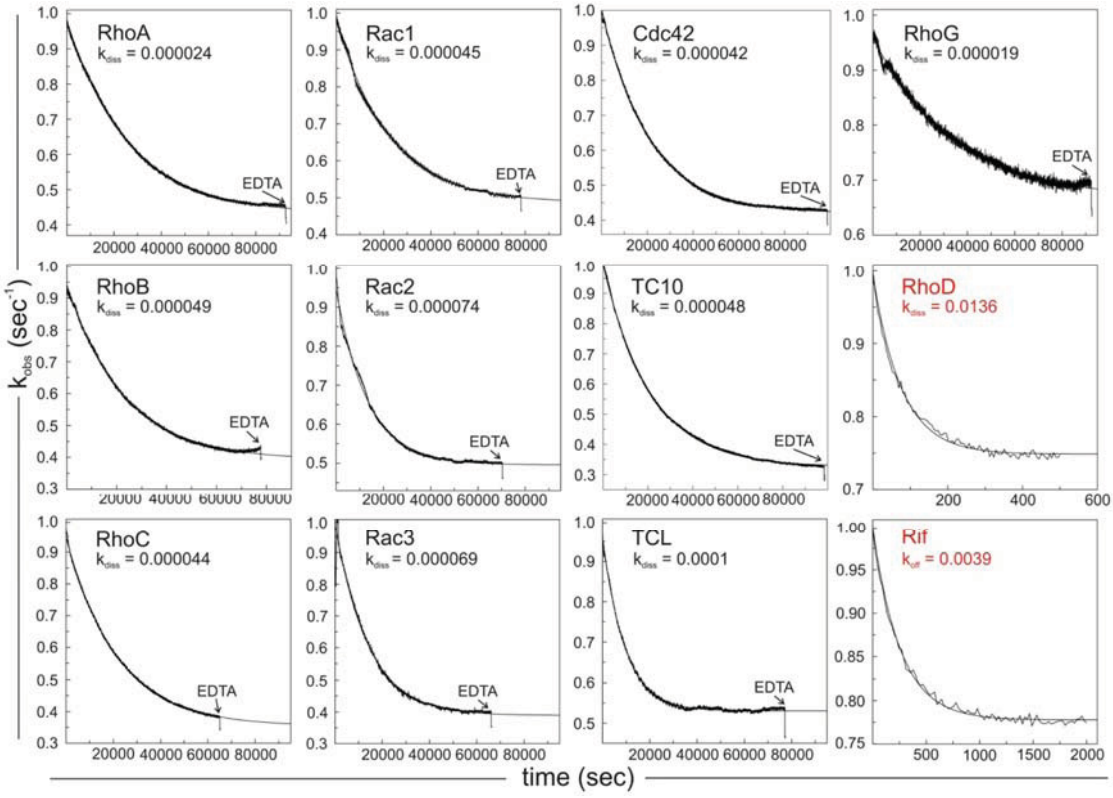
Supplementary Figure S1: Multiple sequence alignment of the G domain of the Rho protein family. (**A**) Rho protein sequences were aligned using ClustalW program and the alignment was edited by GeneDoc. The conserved signatures of the Rho proteins are designated as G1 (P loop binds), G2 (switch I), G3 (switch II), G4 (guanine base binding) and G5 (guanine base binding). Conserved residues in G1-G5 motifs are highlighted in white and red background (X). Residues in white and black background (X) may be responsible for the fast nucleotide dissociation from RhoD and Rif. Residues in white and green background (X) are most likely responsible for the slow nucleotide association with RhoB, Rac2 and TC10. Catalytic residues that are not conserved in Rnd1, Rnd2 and Rnd3 proteins are highlighted in white and blue background (X).Alternative name of the proteins are summarized in the **supplementary** table S1. (**B**) Phylogenetic tree of the G domain of the Rho protein family. Phylogenetic diagram was generated using program MEGA (http://www.megasoftware.net/) and shows the exceptional status of RhoD, Rif, TTF and the Rnd proteins.



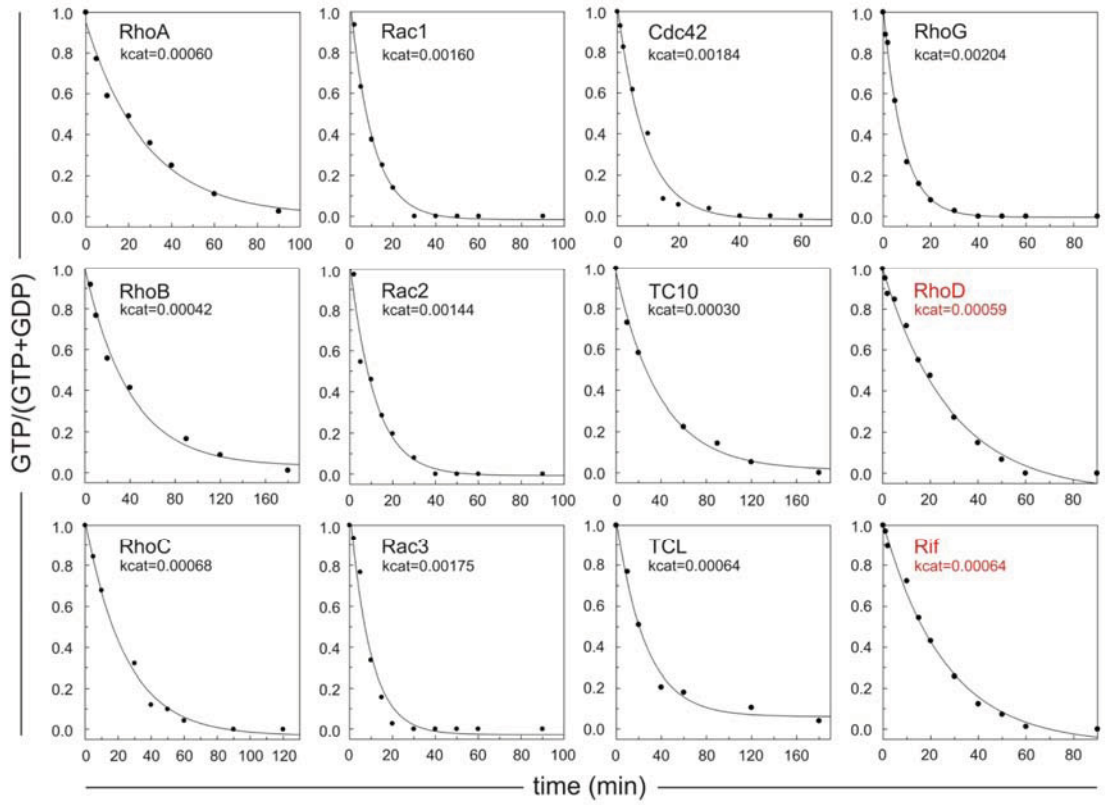
Supplementary Figure S2: Multiple sequence alignment of the nucleotide binding residues of Rho proteins family. (**A**) Nucleotide binding residues of Rho proteins sequences were aligned using ClustalW program and the alignment later was edited by GeneDoc. Invariable residues are highlighted in white and black background (X), highly conserved residues (80%) in white and grey background (X) and conserved residues (60%) in black and grey background (X). (**B**) Phylogenetic tree of the nucleotide binding residues of the Rho protein family. Phylogenetic diagram was generated using program MEGA (http://www.megasoftware.net/) and shows the distinct status of Rif, RhoD and the Rnd proteins regarding in this case only active site of Rho GTPases.



Supplementary Figure S3: Real-time monitoring of RhoA and nucleotide association kinetics. (A) Kinetics of association between florescent labelled mantGDP (0.1 µM) and different concentration (0.05 - 2 µM) of nucleotide free RhoA were measured. Data were collected using stopped-flow instrument with mant fluorescence as a signal of binding. Observed rate constants (k_{obs}) of associations were by single exponential fitting of individual curves with increasing protein concentrations. (B) The association rate constants (k_{ass}) of 12 Rho proteins for mantGDP binding were calculated from the linear regression of the k_{obs} values plotted against the concentration of the nucleotide free Rho proteins. The association rate (k_{ass}) is represented by the slope of regression lines. All k_{ass} values are summarized in Table 1. Association of mantGDP to nucleotide free GTPases were carried out at 25°C in 30 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 10 mM KH_2PO_4/K_2HPO_4 and 3 mM DTT.



Supplementary Figure S4: Intrinsic nucleotide dissociation of Rho GTPases. The intrinsic nucleotide exchange of Rho GTPases were measured at 25°C in 30 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 10 mM KH_2PO_4/K_2HPO_4 and 3 mM DTT by the monitoring of mantGDP dissociation from Rho•mantGDP (0.1 µM) after the addition of 20 µM unlabeled GDP. The intrinsic nucleotide exchange reaction was completed after addition of 20 mM EDTA (arrow). Observed dissociation rate constants of intrinsic (k_{diss}) were obtained by single exponential fitting of the data. All k_{diss} values are summarized in Table 1.



Supplementary Figure S5: Intrinsic GTP hydrolysis of Rho GTPases. The intrinsic GTPase reaction was measured by mixing 70 µM nucleotide free Rho protein with 50 µM GTP and incubating the reaction mixture at 25ºC in 30 mM Tris-HCl pH 7.5, 10 mM KH_2PO_4/K_2HPO_4, 10 mM $MgCl_2$, 3 mM DTT. Samples have been taken at different time intervals, immediately frozen in liquid nitrogen and later analyzed for the amount of guanine nucleotide by HPLC assay in a time-dependent manner. Relative GTP contents were calculated from the ratio of (GTP)/(GDP+GTP). Intrinsic catalytic rate constants (k_{cat}) of different proteins were obtained by single exponential fitting of the data (see also Table 1).

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Mechanistic Insights into Specificity, Activity, and Regulatory Elements of the Regulator of G-protein Signaling (RGS)-containing Rho-specific Guanine Nucleotide Exchange Factors (GEFs) p115, PDZ-RhoGEF (PRG), and Leukemia-associated RhoGEF (LARG)*[S]

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The multimodular guanine nucleotide exchange factors (GEFs) of the Dbl family mostly share a tandem Dbl homology (DH) and pleckstrin homology (PH) domain organization. The function of these and other domains in the DH-mediated regulation of the GDP/GTP exchange reaction of the Rho proteins is the subject of intensive investigations. This comparative study presents detailed kinetic data on specificity, activity, and regulation of the catalytic DH domains of four GEFs, namely p115, p190, PDZ-RhoGEF (PRG), and leukemia-associated RhoGEF (LARG). We demonstrate that (i) these GEFs are specific guanine nucleotide exchange factors for the Rho isoforms (RhoA, RhoB, and RhoC) and inactive toward other members of the Rho family, including Rac1, Cdc42, and TC10. (ii) The DH domain of LARG exhibits the highest catalytic activity reported for a Dbl protein till now with a maximal acceleration of the nucleotide exchange by 10^7-fold, which is at least as efficient as reported for GEFs specific for Ran or the bacterial toxin SopE. (iii) A novel regulatory region at the N terminus of the DH domain is involved in its association with GDP-bound RhoA monitored by a fluorescently labeled RhoA. (iv) The tandem PH domains of p115 and PRG efficiently contribute to the DH-mediated nucleotide exchange reaction. (v) In contrast to the isolated DH or DH-PH domains, a p115 fragment encompassing both the regulator of G-protein signaling and the DH domains revealed a significantly reduced GEF activity, supporting the proposed models of an intramolecular autoinhibitory mechanism for p115-like RhoGEFs.

The small GDP/GTP-binding proteins (GTPases)[4] of the Rho family are key regulators in a multitude of cellular processes (1, 2). Like almost all GTPases, the Rho proteins function as binary switches, cycling between an inactive GDP-bound state and an active GTP-bound state (3, 4). In response to diverse extracellular stimuli, guanine nucleotide exchange factors (GEFs) catalyze the exchange of bound GDP for cellularly abundant GTP in their cognate GTPase substrates and thereby initiate Rho signaling cascades (5–12).

The common structural module of Dbl family GEFs (69 members known), which is responsible for the nucleotide exchange activity, consists of a Dbl homology (DH) domain and an adjacent pleckstrin homology (PH) domain C-terminal to the DH domain (9, 11). The DH domain makes extensive contacts with switch I and II regions of RhoGTPases and contains virtually all the residues required for substrate recognition, binding, and guanine nucleotide exchange (9, 11)). The nearly invariant domain organization of the DH-PH tandem in all members of the Dbl family presumes a conserved function for the PH domain. However, a clear role of the tandem PH domain has not yet been established. In some cases, the PH domain seems to facilitate the catalytic activity of the DH domain. For example, residues within the PH domain of Dbs interact directly with the bound RhoGTPase (13, 14) and enhance nucleotide exchange on Cdc42 and RhoA (14, 15). A similar scenario has been reported for the PH domains of PDZ-RhoGEF/GTRAP48 (hereafter called PRG) and leukemia-associated RhoGEF (LARG) (16, 17). Conversely, the PH domains of son of sevenless homolog 1 (Sos1) and Trio-N appear to inhibit nucleotide exchange on Rac1 and RhoG, respectively (18, 19). The tandem PH domain of other GEFs, including Tiam1, Intersectin1 (ITSN1), and Pem-2/Collybistin II, has been shown not to contact the respective GTPase at all (13, 20, 21).

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[4] The abbreviations used are: GTPase, GDP/GTP-binding protein; GEF, guanine nucleotide exchange factor; PRG, PDZ-RhoGEF; LARG, leukemia-associated RhoGEF; RGS, regulator of G-protein signaling; DH, Dbl homology; PH, pleckstrin homology; ITSN1, Intersectin1; 1,5-I-AEDANS (AEDANS), *N*-(iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid; GppNHp, guanosine 5′-(β,γ-imido)triphosphate; mant, methylanthraniloyl; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; GDPγS, guanosine 5′-3-*O*-(thio)-triphosphate; fRhoA, fluorescent RhoA; PDZ, postsynaptic density-95, discs large, and zona occludens-1.

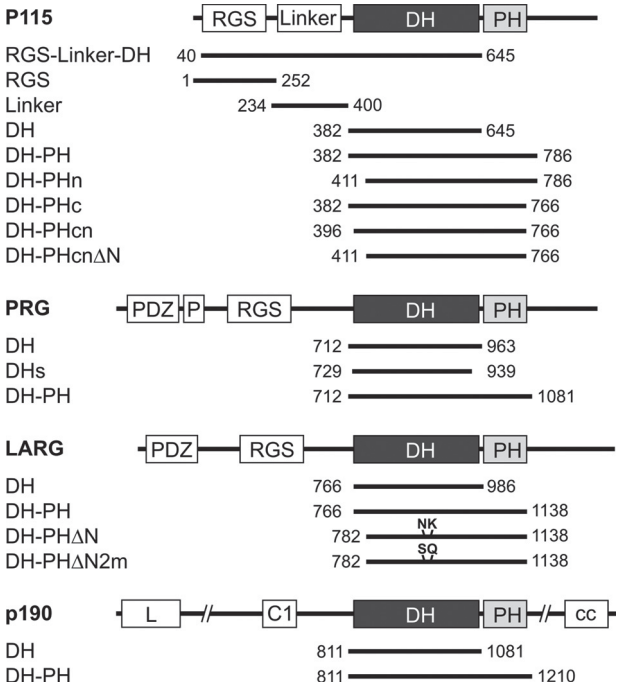

FIGURE 1. **Schematic representation of domain organization and different constructs of p115, p190, PRG, and LARG used in this study.** The *numbers* indicate the N- and C-terminal amino acids of the respective constructs. DH-PHn, DH-PHc, and DH-PHcn are shorter variants at the N and C termini of p115 DH-PH that are equivalent to LARG DH-PH (see supplemental Fig. S2A). DH-PHcnΔN and DH-PHΔN are N-terminally deleted variants of p115 and LARG and equivalent to each other. DH-PHΔN2m contains two point mutations at positions Asn^{946} and Lys^{949} that are substituted by Ser and Gln, the corresponding residues in p115. *C1*, cysteine-rich region; *cc*, coiled coil; *L*, leucine-rich; *P*, proline-rich.

In contrast to the conservation of the DH-PH tandem, the GEFs of the Dbl family also exhibit a variety of functional domain compositions and domain organizations (7, 8, 11, 22), which link their GEF activity to specific signaling events. Interesting examples in this regard are regulator of G-protein signaling (RGS) domain-containing RhoGEFs, such as p115, PRG, and LARG. The RGS domain at the N terminus of p115 directly links the heterotrimeric G proteins $G\alpha_{12/13}$ to RhoA regulation and acts as a GTPase-activating protein for $G\alpha_{12/13}$ (23, 24). The association of p115 with $G\alpha_{12}$ and $G\alpha_{13}$ has been suggested to activate its GEF function toward Rho proteins (25–28). A similar regulatory model has been proposed for PRG and LARG (29–36). Recently, Zheng *et al.* (37) provided direct biochemical evidence for an autoinhibitory RGS-mediated regulation of the DH domain.

Despite intensive research, there is little comparative analysis of these RGS-containing GEFs available. Thus, we purified different protein domains of p190, p115, PRG, and LARG (Fig. 1) and characterized them functionally regarding their specificity, activity, and regulation with respect to each other. We measured their effects on the DH-catalyzed nucleotide exchange of RhoGTPases by means of fluorescence spectroscopy utilizing both GTPases loaded with fluorescently labeled guanine nucleotides (38) and a fluorescent RhoA itself (RhoA(V33C)-AEDANS) (this study). p190, a Rho-specific GEF (39), was used as a control. Our results suggest that the PH domains of PRG and p115 participate in the DH-catalyzed exchange but not in the association reaction and that the N-terminal regions of p115 possibly represent an autoregulatory module. In addition, we demonstrate that these four GEFs are specific for the Rho isoforms (RhoA, RhoB, and RhoC) and are able to catalyze their very slow intrinsic nucleotide dissociation up to 7 orders of magnitude beyond the capability of any other GEF reported so far.

EXPERIMENTAL PROCEDURES

Constructs—Constructs of human p115 DH (residues 382–645), DH-PH (residues 382–786), DH-PHc (residues 382–766), DH-PHcn (residues 396–766), DH-PHcnΔN (residues 411–766), RGS (residues 1–252), RGS-Linker (residues 40–400), Linker (residues 234–400), RGS-Linker-DH (residues 40–645), and Linker-DH (residues 234–645); murine p190 DH (residues 811–1081) and DH-PH (residues 811–1210); human PRG DH (residues 712–963), DHs (residues 729–939), and DH-PH (residues 712–1081); human LARG DH (residues 766–986), DH-PH (residues 766–1138), and DH-PHΔN (residues 782–1138); and C-terminal truncated human RhoA (residues 1–181), RhoB (residues 1–181), RhoC (residues 1–181), Cdc42 (residues 1–178), Rac1 (residues 1–184), and TC10 (residues 1–193) were amplified by standard PCR and cloned in pGEX-4T1 and pGEX-4T1-Ntev vector, respectively. Point mutations in RhoA (residues 1–181) at position Val^{33} to Cys and in LARG DH-PHΔN at positions Asn^{946} to Ser and Lys^{949} to Gln were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

Proteins—All proteins were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* BL21(DE3)pLyS or alternatively CodonPlusRIL, isolated in a first step by affinity chromatography on a glutathione-Sepharose column, and purified after proteolytic cleavage of GST in a second step by size exclusion chromatography (Superdex S200) as described (38). GTPases either in complex with non-labeled nucleotides (GDP or GppNHp), with fluorescent nucleotides (methylanthraniloyl-GDP (mantGDP) or mantGppNHp), or without nucleotide (the nucleotide-free form) were prepared as described (38). Purified proteins were snap frozen in liquid nitrogen and stored at −80 °C.

Fluorescence Labeling of RhoA with AEDANS—For coupling the AEDANS fluorescence reporter group, purified GDP-bound RhoA(V33C) was transferred to buffer containing 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM ascorbate by repeated dilution and ultrafiltration steps. Protein was then incubated overnight with a 10-fold excess of 1,5-I-AEDANS (Sigma). It should be mentioned that other fluorescence reporter groups, such as fluorescein, Alexa Fluor, and pyrene, were not tested because they are proven to be less environmentally sensitive or result in precipitation of the proteins after labeling (40). The reaction was stopped by adding of dithioerythritol in excess. Unbound AEDANS was removed by sequential dilution and ultrafiltration steps. The efficiency of the labeling reaction was analyzed by mass spectrometry.

Fluorescence Measurements—All fluorescence measurements were performed in 30 mM Tris/HCl, pH 7.5, 10 mM K_2HPO_4/KH_2PO_4, pH 7.5, 5 mM $MgCl_2$, 3 mM DTT at 25 °C. The mant-GDP dissociation rates from RhoA (0.1 μM) were measured in

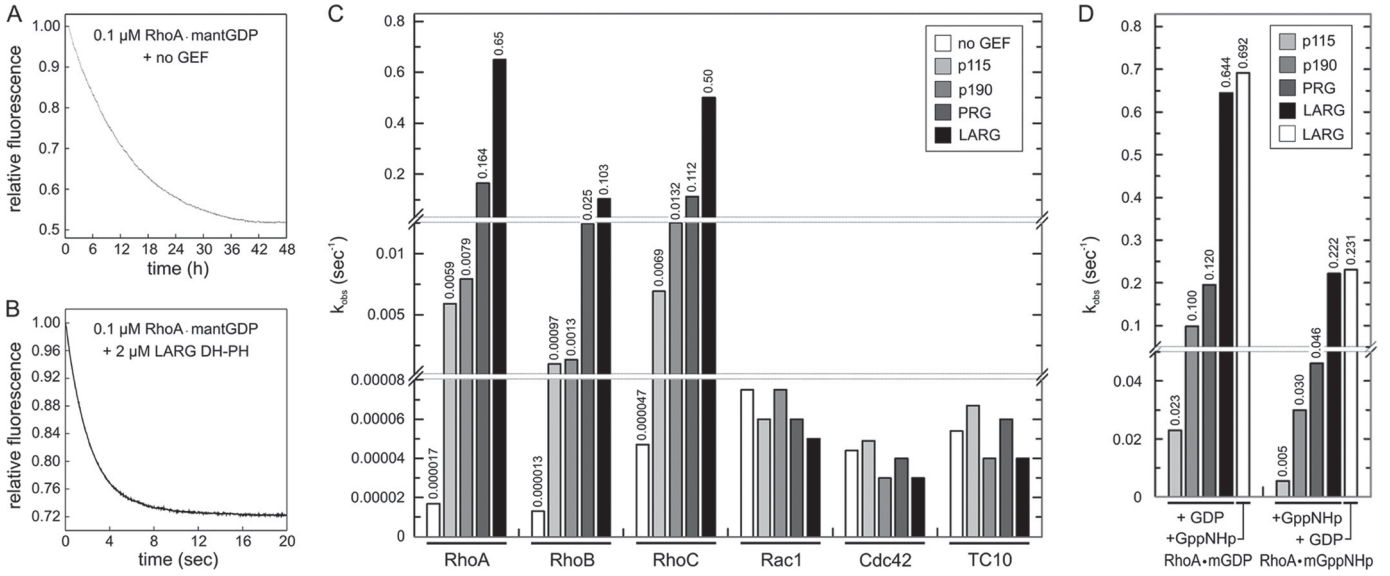

FIGURE 2. **Rho specificity of p115, p190, PRG, and LARG.** *A* and *B*, DH-PH catalyzes the very slow intrinsic nucleotide exchange reaction by several orders of magnitude. The mantGDP dissociation from 0.1 μM RhoA was monitored after addition of 20 μM unlabeled GDP in the absence (*A*) and in the presence of 2 μM LARG DH-PH (*B*). Note the dimension of the *x axis*, which is in hours in *A* and in seconds in *B*, visualizing a rate acceleration of more than 38,000-fold. *C*, Rho isoform specificity of p115, p190, PRG, and LARG. The observed rate constants (k_{obs}) of both intrinsic and DH-PH-catalyzed reactions of different GTPases were obtained by single exponential fitting of the data. The k_{obs} values were determined using 0.1 μM mantGDP-bound GTPases (RhoA, RhoB, RhoC, Rac1, Cdc42, and TC10) and 20 μM non-fluorescent GDP in the mantGDP dissociation catalyzed by four different DH-PH proteins (2 μM each). *D*, DH-PH-catalyzed nucleotide exchange is independent of the type of bound nucleotide. GEF-catalyzed mantGDP and mantGppNHp dissociation from RhoA was monitored using 0.1 μM mant-nucleotide-loaded RhoA (RhoA-mantGDP or RhoA-mantGppNHp) and 20 μM non-fluorescent nucleotide (GDP or GppNHp) in the presence of 10 μM DH-PH domain of p115 or p190 or 2 μM DH-PH domain of PRG or LARG. Note that a 5-fold lower concentration of LARG and PRG has been used compared with the experiments with p190 and p115. Moreover, the LARG-catalyzed mant-nucleotide dissociation was measured in the presence of excess amounts of both GDP and GppNHp (*white bar*). The observed rate constants (k_{obs}) were obtained by single exponential fitting of the data. For convenience, the exact k_{obs} values are given as *numbers* above the *bars* in C and D.

the absence and presence of different amounts of respective DH proteins as described previously for Rac proteins (38, 41, 42). Fast kinetics (<1000 s) were performed with an Applied Photophysics (SX18MV) or with a Hi-Tech Scientific (SF-61) stopped-flow instrument, respectively. The excitation wavelengths were 366 nm for mant and 350 nm for AEDANS. Emission was detected through a cutoff filter of 408 nm for both mant and AEDANS. Slow kinetics (>1000 s) were measured on a PerkinElmer Life Sciences spectrofluorometer (LS50B) or on a FluoroMax spectrofluorometer (SPEX Instruments, Edison, NJ), respectively, using an excitation wavelength of 366 nm for mant and 350 nm for AEDANS and an emission wavelength of 450 nm for mant and 490 nm for AEDANS. Data were processed as described before (38, 40).

RESULTS

DH-PH Tandem Determines GEF Specificity—Considerable advantages in the investigation of the GEF-accelerated nucleotide exchange reaction are provided by fluorescence spectroscopy (38, 41, 43). In this method, the displacement of fluorescent mantGDP from RhoA in the presence of an excess amount of non-fluorescent GDP resulted in a significant change in fluorescence intensity over the time course of the reaction (Fig. 2*A*). The very slow intrinsic nucleotide dissociation rate (1.7×10^{-5} s^{-1}) was efficiently accelerated 3.8×10^4-fold (with an observed rate constant or k_{obs} of 0.65 s^{-1}) when 2 μM of the DH-PH domain of LARG was mixed in the stopped-flow apparatus with 0.1 μM RhoA-mantGDP and 20 μM GDP (Fig. 2*B*). For comparison, the GEF activities of p115, p190, and PRG were measured under the same condition (Fig. 2*C*). All three GEFs were less efficient in the acceleration of nucleotide dissociation compared with LARG. Although PRG DH-PH accelerated the nucleotide exchange 9.6×10^3-fold, which was only 4 times slower than LARG, p115 and p190 only showed a 0.5×10^3- and 0.3×10^3-fold acceleration, which was 82- and 110-fold slower than LARG, respectively. It has been shown that PRG is not only a Rho-specific GEF but to a certain extent also active on Cdc42 (16). Therefore, we measured the rate of mantGDP dissociation from Rac1, Cdc42, and TC10 in the absence and in the presence of the DH-PH domain of p115, p190, PRG, or LARG, respectively. As shown in Fig. 2*C*, we did not observe any significant changes in the nucleotide dissociation rates of these GTPases by p115, p190, PRG, or LARG. The RacGEF Tiam1 (41, 42) and the Cdc42GEF Asef (44) were used as positive controls (supplemental Fig. S1). This clearly emphasizes the specificity of these GEFs for RhoA. To complete the scenario, we next determined the efficiency of p115, p190, PRG, and LARG on the exchange of mantGDP bound to RhoB and RhoC GTPases, which share 84 and 92% sequence identity with RhoA, respectively. We found that these two Rho isoforms together with RhoA are specific substrates for the investigated GEFs (Fig. 2*C*). Their intrinsic nucleotide dissociation rates (1.3×10^{-5} s^{-1} for RhoB and 4.7×10^{-5} s^{-1} for RhoC) were also accelerated up to 4 orders of magnitude in the presence of a 2 μM concentration of the respective DH-PH (Fig. 2*C*).

RhoGEF-catalyzed Exchange Reaction Is Independent of Type of Incoming Nucleotide—The activation process of small GTPases is an intensively studied issue. However, it remains unclear how GEFs approach the inactive GDP-bound GTPase

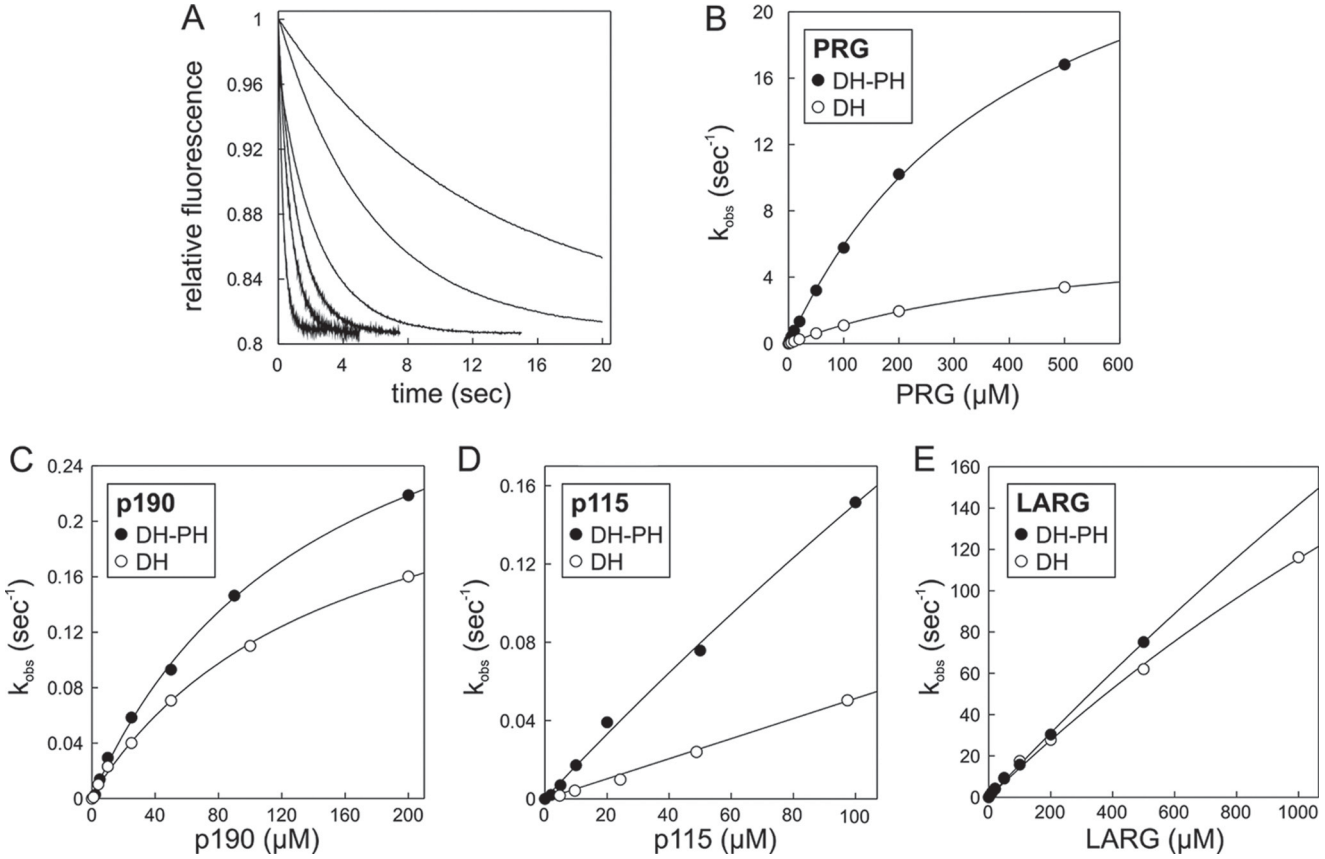

FIGURE 3. **Kinetics of catalyzed nucleotide dissociation reaction of RhoA by RhoGEFs PRG, p190, p115, and LARG using fluorescent nucleotides.** *A*, kinetics of mantGDP dissociation from RhoA (0.1 μM) were measured in the presence of 20 μM non-fluorescent GDP and increasing concentrations of the DH-PH domain of PRG (1, 2, 5, 10, 20, and 50 μM) under the same condition as in Fig. 2. Observed rate constants (k_{obs}) of the respective data were obtained by single exponential fitting. The dependence of the k_{obs} values for the mantGDP dissociation on the concentrations of the DH-PH (*closed circles*) and the DH (*open circles*) domains of PRG (*B*), p190 (*C*), p115 (*D*), and LARG (*E*) was fitted to a hyperbolic curve to obtain the kinetic parameters of the GEF-catalyzed nucleotide dissociation from RhoA.

and which role the switch regions have in this context. To address this question, we measured the GEF-catalyzed mantGDP and mantGppNHp dissociation from RhoA in the presence of an excess amount of GDP or GppNHp (a non-hydrolyzable analog of GTP), respectively. The Dbl proteins p115, p190, PRG, and LARG were able to catalyze the mantGppNHp dissociation from RhoA, but the efficiency was 3–4-fold lower than that for mantGDP (Fig. 2*D*). Moreover, there was no difference whether the incoming nucleotide was GDP or GppNHp as shown for the LARG-catalyzed reaction (Fig. 2*D*). These data suggest that the conformation of the switch regions of RhoA plays a rather marginal role in GEF recognition, and the exchange machinery functions regardless of the type of nucleotide.

DH Domain Is a Highly Efficient Catalytic Machine—To obtain the maximal rates of the catalyzed nucleotide exchange reaction and a rough estimate of the catalytic efficiency of the complexes between RhoA-mantGDP and the DH-PH domains of p115, p190, PRG, or LARG, we performed single turnover stopped-flow measurements under the same conditions as described above. As shown in Fig. 3*A*, the decrease in fluorescence, which corresponds to the catalyzed mantGDP dissociation from RhoA, occurred incrementally faster with the addition of increasing amounts of the DH-PH domain of PRG (1–50 μM). Corresponding k_{obs} values were plotted against the varied DH-PH concentrations (Fig. 3*B*, *closed circles*). The kinetic parameters of the PRG-accelerated nucleotide dissociation from RhoA were estimated from the hyperbolic kinetics with a K_m of 425.7 μM and a k_{max} of 31.3 s^{-1} as described elsewhere (38). As in the case of PRG, a complete saturation was also not achieved for the reaction catalyzed by the p190 DH-PH tandem (Fig. 3*C*, *closed circles*). Nevertheless, p190 appears to have a much lower k_{max} (0.37 s^{-1}) and a higher K_m (143.5 μM) as compared with PRG DH-PH.

For LARG DH-PH, which also belongs to the RGS-containing Dbl protein family (Fig. 1), we obtained the highest catalytic activity of 75 s^{-1} at a protein concentration of 500 μM, which also was still far below saturation (Fig. 3*E*, *closed circles*). Similarly, the data obtained for p115 indicate that K_m and k_{max} must be far beyond 100 μM and 0.15 s^{-1}, respectively (Fig. 3*D*, *closed circles*). Hence, as the single concentration measurements already indicated, LARG and PRG are 2 orders of magnitude more efficient catalysts of the nucleotide exchange reaction as compared with p115 and p190. The overall acceleration of approximately 0.88×10^4-fold by p115, 2.17×10^4-fold by p190, most remarkably 1.84×10^6-fold by PRG, and at least 4.70×10^6-fold by LARG was calculated from the ratio of the respective k_{max} or maximal k_{obs} values and the intrinsic dissociation rate of mantGDP (1.7×10^{-5} s^{-1}; Fig. 2*A*). This clearly demonstrates that the GEFs of the Dbl family proteins are able

to catalyze the nucleotide exchange of RhoGTPases as efficiently as was reported previously for the GEFs of Ran, Ras, and Rab (45–47).

PH-assisted Exchange Reaction of p115 and PRG but Not of p190 and LARG—The majority of Dbl family proteins comprise a characteristic tandem DH-PH organization, suggesting that the PH domain may provide an essential, conserved function in the regulation of DH domain activity. Therefore, we scrutinized the influence of the PH domains of the four RhoGEFs on the DH-catalyzed nucleotide exchange. Similarly to the tandem DH-PH, hyperbolic dependences of the mantGDP dissociation rate on DH concentration were observed for PRG and p190 (Fig. 3, *B* and *C*). They were saturated at a k_{max} value of 6.9 (PRG) and 0.28 s^{-1} (p190) and an apparent K_m value of 516.7 (PRG) and 151.4 μM (p190), respectively (Fig. 3, *B* and *C*, *open circles*). It is important to note that a shorter segment of PRG DHs (residues 729–939), which has been reported to be inactive (16), could not be analyzed because it turned out to be insoluble (data not shown). A possible reason may be the truncated α13 at the C terminus (supplemental Fig. S2A). The PH domain of p115 also appeared to influence considerably the activity of its DH domain as the rate of nucleotide dissociation was ~4-fold lower for each particular concentration of the corresponding protein constructs (Fig. 3*D*, *open circles*). However, because the kinetic parameters for the nucleotide exchange reaction of p115 DH on RhoA-mantGDP could not be determined, it remains unclear whether this effect results either from reduced activity or from reduced affinity. Also only minor differences were observed for the nucleotide exchange between the DH and DH-PH for LARG, indicating that the tandem PH domain of this GEF influences the acceleration of the nucleotide exchange reaction of RhoA only marginally (Fig. 3*E*, *open circles*).

Fluorescent RhoA Allows Monitoring RhoA-GDP Association of RhoGEFs—Although the use of mantGDP proved to be very useful for the elucidation of GEF-catalyzed nucleotide dissociation from RhoGTPases, it does not enable monitoring of events upon RhoGEF association. Therefore, we set out to extend our technical capacity by developing a method that enables us to measure the binding kinetics of RhoA-GDP with GEFs in real time. An alternative, fluorescence-based approach is the introduction of reporter groups into the interacting partner, RhoA or the DH domain of the RhoGEFs. Labeling of small GTPases with fluorescent reporter groups has so far only been used to study the interaction of Cdc42 with an effector (48) and the mechanism of NTF2-mediated Ran transport into the nucleus (49), with C-terminally dansyl-labeled Rab to study the interaction with the prenyltransferases (50), and with H-Ras and Rap1 to measure GTPase-activating protein binding (40).

Specific attachment of fluorophores on protein surfaces can be achieved via the modification of thiol groups of cysteines. C-terminally truncated RhoA (residues 1–181) contains five cysteines, but none of them is accessible from the solvent according to the structures of RhoA-GDP (51) and RhoA-GTPγS (52). This was verified by performing an Ellman reaction with GDP-bound and GppNHp-bound RhoA proteins, including RhoA(C20S) as a control, using Ellman's reagent (5,5′-dithiobis(nitrobenzoic acid); Sigma) (40, 53). We then inspected the structures of RhoA in complex with the DH-PH domains of PRG (16, 54, 55) and LARG (17) to identify the residues on the RhoA surface that are close to or at the edge of the binding interface but do not participate significantly in the interaction with DH-PH (Fig. 4*A*). From nine freely accessible residues, we chose the conserved Val^{33} (supplemental Fig. S2A) that was replaced by cysteine and labeled with the fluorescence reporter group AEDANS (see "Experimental Procedures"). AEDANS-labeled fluorescent RhoA(V33C), which is called, for simplicity, fRhoA, notably showed an incremental increase in fluorescence in the presence of increasing amounts of p115 DH-PH (Fig. 4*B*). This was not observed when we applied to fRhoA a Rac1-specific DH-PH of Tiam1 instead of p115 (data not shown). A rate constant for the association (k_{on}) of 1.186×10^5 s^{-1} M^{-1} was calculated by plotting k_{obs} values of the corresponding binding curves against the p115 DH-PH concentrations (Fig. 4*C* and supplemental Fig. S3, filled circles). The dissociation rate constant (k_{off}) was determined by displacing fRhoA-GDP from its complex with p115 DH-PH in the presence of unlabeled, nucleotide-free RhoA, a reaction that led to rapid decrease in fluorescence (Fig. 4*D* and supplemental Fig. S3, black line). Nucleotide-free RhoA has a much higher affinity for the GEFs compared with the nucleotide-bound forms of the GTPases (56).[5] The obtained dissociation rate constant (k_{off}) of 1.7 s^{-1} divided by the k_{on} value enabled us to calculate a dissociation constant (K_d) of 14.5 μM for the fRhoA/GDP interaction with the p115 DH-PH proteins.

LARG Association with RhoA-GDP Is Strikingly Faster than p115—With fRhoA, we now have an attractive technique that enables us to better understand both the differential RhoA binding characteristics of the RhoGEFs and the role of the PH domains in the exchange reactions. As shown on Figs. 2 and 3, the largest differences in exchange efficiency and involvement of PH domain were observed for LARG and p115. Therefore, we determined the individual rate constants for the interaction of fRhoA-GDP with the DH and DH-PH domains of these two GEFs. Fig. 4*C* shows that LARG associated with fRhoA-GDP 15-fold faster than p115 independently of the presence or absence of the PH domain. Interestingly, this was not the case for the dissociation reaction (Fig. 4*D*). The k_{off} values are similar for LARG DH and DH-PH but vary about 2-fold between p115 DH and DH-PH (Fig. 4*D*). This is consistent with our data on the involvement of the PH domain of p115 but not of LARG in nucleotide exchange catalysis (Fig. 3, *D* and *E*). Moreover, these data strongly suggest that the efficiency of LARG in catalyzing the mantGDP dissociation is in fact attributed to its faster association with fRhoA-GDP.

Short N-terminal DH Segment Is Critical for Binding and Catalysis—The results described above demonstrate clearly different binding capacities and catalytic efficiencies of the RhoGEFs investigated but did not reveal which regions in the DH domain are responsible for their association with the GDP-bound RhoA. To shed light on the molecular basis of the observed differences, we inspected individual amino acids of the DH domains of LARG, PRG, p190, and p115. We sorted out

[5] Z. Guo and M. R. Ahmadian, unpublished data.

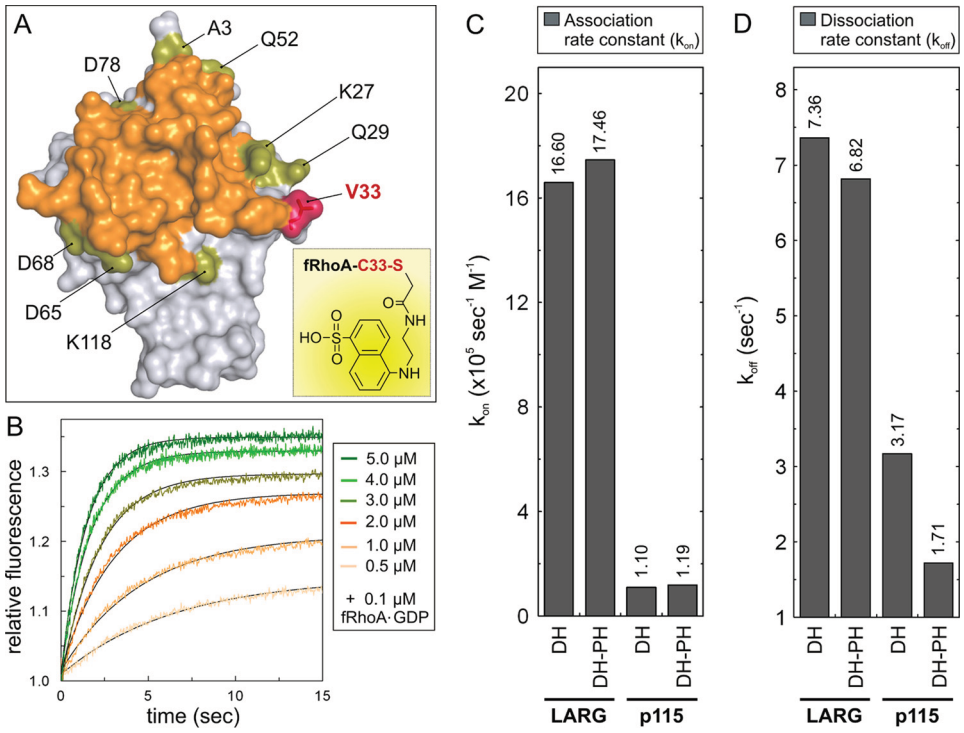

FIGURE 4. **Real time monitoring of RhoGEF interactions with GDP-bound fRhoA.** *A*, RhoA labeling strategy with the fluorescence reporter group AEDANS (*inset*). The van der Waals surface of nucleotide-free RhoA from the LARG DH-PH complex (17) (Protein Data Bank code 1X86) shows the solvent-accessible surrounding residues (*green*) around the interaction surface of LARG (*orange*). Valine 33 (*V33*) of RhoA substituted by cysteine and labeled with AEDANS (fRhoA) is shown in *red*. *B*, fRhoA allows monitoring of the RhoGEF association in real time. Rapid mixing of increasing p115 DH-PH concentrations (0.5–5 μM) with fRhoA-GDP (0.2 μM) resulted in an incremental increase in fluorescence corresponding to the association reaction. *C*, the association rate constants (k_{on}) of fRhoA-GDP binding to the DH and DH-PH proteins of LARG and p115, respectively, clearly revealed differences in the binding properties of the two RhoGEFs. *D*, the dissociation rate constant (k_{off}) of the DH and DH-PH proteins of LARG and p115, respectively, displaced from the fRhoA-GDP complex in the presence of excess amounts of unlabeled, nucleotide-free RhoA revealed an impact of p115 PH domain on the GEF dissociation kinetics. The kinetic data are shown in supplemental Fig. S3. The dissociation constant (K_d) was calculated from the kinetic parameters of dissociation and association reactions by the equation $K_d = k_{off}/k_{on}$. For convenience, the exact k_{on} and k_{off} values are given as *numbers* above the *bars* in *C* and *D*, respectively.

identical residues and selected variable residues between p115 *versus* LARG and PRG using a multiple sequence alignment of the DH domains (supplemental Fig. S2A). Based on the crystal structures of RhoA in complex with DH-PH of PRG (16) and of LARG (17), we further selected solvent-exposed residues that are close to or part of the interacting interface and identified eight potential residues (supplemental Fig. S2A, red underlined). Structural analysis of these amino acids interestingly showed that among these eight residues six are close to the switch I region and four of these, namely Asn^{768}, Asp^{770}, Arg^{775}, and Gly^{780}, are clustered at the very N-terminal segment (Fig. 5*A*).

Considering the fact that the crystal structures of RhoA-LARG and RhoA-PRG complexes are nucleotide-free complexes, it is rather tempting to speculate that these eight residues may play a role in the DH association with GDP-bound RhoA. Thus, we first generated a deletion mutant of the DH-PH of LARG (DH-PHΔN) lacking the N-terminal segment with its four putative association-determining residues Asn^{768}, Asp^{770}, Arg^{775}, and Gly^{780} (Fig. 5*A*). We measured the properties of DH-PHΔN regarding its association with fRhoA-GDP and its activity in catalyzing the mantGDP dissociation from RhoA in comparison with LARG DH-PH. Fig. 5, *B* and *C*, show that a deletion of 16 amino acids at the N terminus of LARG DH-PH (DH-PHΔN; Fig. 1 and supplemental Fig. S2A) clearly and substantially affected its association efficiency and consequently its catalytic activity. Having partially proven our hypothesis, we mutated two further potential residues in DH-PHΔN, namely Asn^{946} and Lys^{949} to Ser^{946} and Gln^{949}, which are the equivalent residues in p115 (DH-PHΔN2m; Figs. 1 and 5*A* and supplemental Fig. S2A). This protein revealed an 8-fold decrease in association with fRhoA-GDP and 25-fold decrease in catalyzing the mGDP dissociation from RhoA (Fig. 5, *B* and *C*). These data strongly suggest that the association of GTPase and GEF is strongly contributing to the catalytic efficiency of the exchange reaction.

Most recently, an acidic stretch upstream of the N-terminal segment that inhibits the catalytic activity of the PRG DH domain has been identified (37). The corresponding glutamic and aspartic acids are conserved in p115 (supplemental Fig. S2A). To determine the impact of both the acidic region and the N-terminal segment on p115 activity under the same experimental conditions, we set out to adjust its DH-PH domain assembly to the length of LARG DH-PH by shortening it at both termini (supplemental Fig. S2A, underlined sequence). DH-PHc and DH-PHcn revealed marginal changes in their ability to associate with fRhoA-GDP, but their nucleotide exchange activity was unexpectedly reduced (Fig. 5, *B* and *C*). To what extent these regions contribute structurally to the GEF activity remains unclear as all structures of PRG and LARG complexes with RhoA are shorter and do not contain these regions (16, 17, 55).

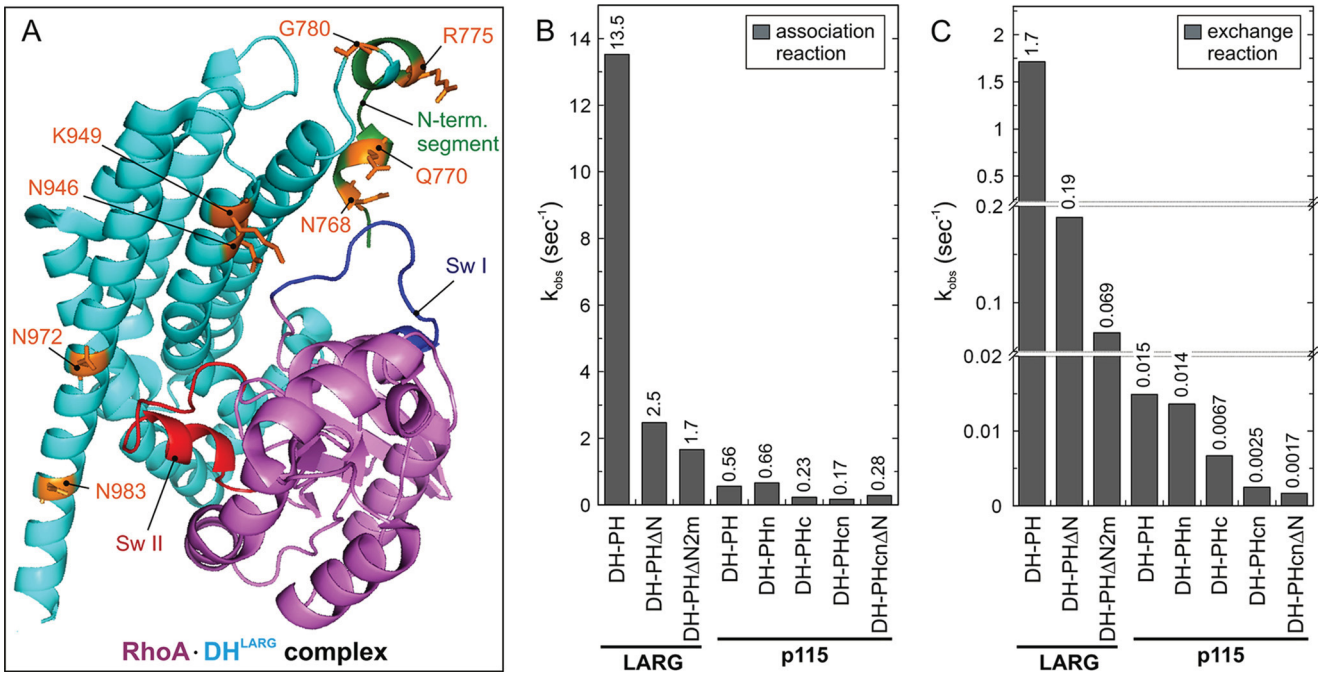

FIGURE 5. **Critical role of N-terminal segment of DH domain in association and nucleotide exchange reactions.** *A*, possible new signatures for the DH function. The crystal structure (17) (Protein Data Bank code 1X86) of the nucleotide-free RhoA (*violet*) in the complex with LARG DH-PH (*turquoise*) highlights eight residues (*orange*) in the DH domain that may be critical for the efficiency of LARG in both associating with GDP-bound RhoA and catalyzing nucleotide dissociation. Four of the eight residues are located in a short peptide called the N-terminal (*N-term.*) segment (*green*). Switch (*Sw*) regions I and II of RhoA and shown in *blue* and *red*, respectively. *B*, the k_{obs} values highlight the association efficiency of the DH-PH variants of LARG and p115 (5 μM, respectively) with 0.2 μM fRhoA-GDP. *C*, the k_{obs} values show the exchange reaction of mantGDP from RhoA (0.1 μM) catalyzed by the DH-PH variants of LARG and p115 (10 μM, respectively). For convenience, the exact k_{obs} values are given as *numbers* above the *bars* in *B* and *C*.

To address the question of what is the impact of the four variable residues within the N-terminal segment on the p115 activity, we analyzed the biochemical properties of p115 DH-PHcnΔN, the fragment equivalent to LARG DH-PHΔN. As shown in Fig. 5, *B* and *C*, neither the rates for association with fRhoA-GDP nor the rates for the catalyzed mantGDP dissociation from RhoA were grossly affected. This is in agreement with our consideration that the N-terminal segment may be an integral element for the catalytic efficiency of LARG and PRG *versus* p115 and p190.

N-terminal RGS-Linker Negatively Controls DH Activity—It appears to be a rule that the Rho family GEFs underlay an autoinhibitory mechanism (37, 57–61). Apart from the catalytic core, which dictates the nucleotide exchange in terms of the DH-PH of RhoGEFs, there are additional domains in the same polypeptides that are essential autoinhibitory elements (37, 57, 58, 60). A G-protein-mediated regulatory principle has been implicated by Sternweis *et al.* (24) for RhoA activation by RGS-containing RhoGEFs. In a recent report, Zheng *et al.* (37) have shown that the RGS domain and a unique sequence motif upstream of the DH domain of PRG (supplemental Fig. S2A) act cooperatively to bind the DH domain and to inhibit its catalytic activity.

To examine a direct modulation of the DH exchange activity by the RGS domain, we measured the catalytic activity of purified p115 RGS-Linker-DH protein under the same condition as described above. As shown in Fig. 6, the GEF activity of the RGS-Linker-DH was 28-fold reduced compared with the isolated DH domain (Fig. 6). Because such an inhibition of the GEF activity strongly suggests an autoinhibitory effect of the DH

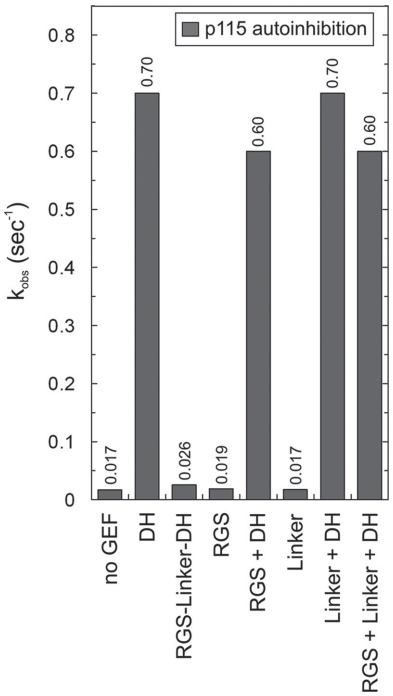

FIGURE 6. **RGS-Linker-mediated autoinhibition of p115 DH Activity.** The effects of various p115 domains on the intrinsic and the DH-catalyzed mant-GDP dissociation from RhoA were measured under the same conditions as in Fig. 2. The following protein concentrations were used: 0.1 μM RhoA-mant-GDP, 1 μM DH, 1 μM RGS-Linker-DH, 10 μM RGS, and 10 μM Linker. The observed rate constants (k_{obs}) of both intrinsic and catalyzed reactions were obtained by single exponential fitting of the data. For convenience, the exact k_{obs} values are given as *numbers* above the *bars*.

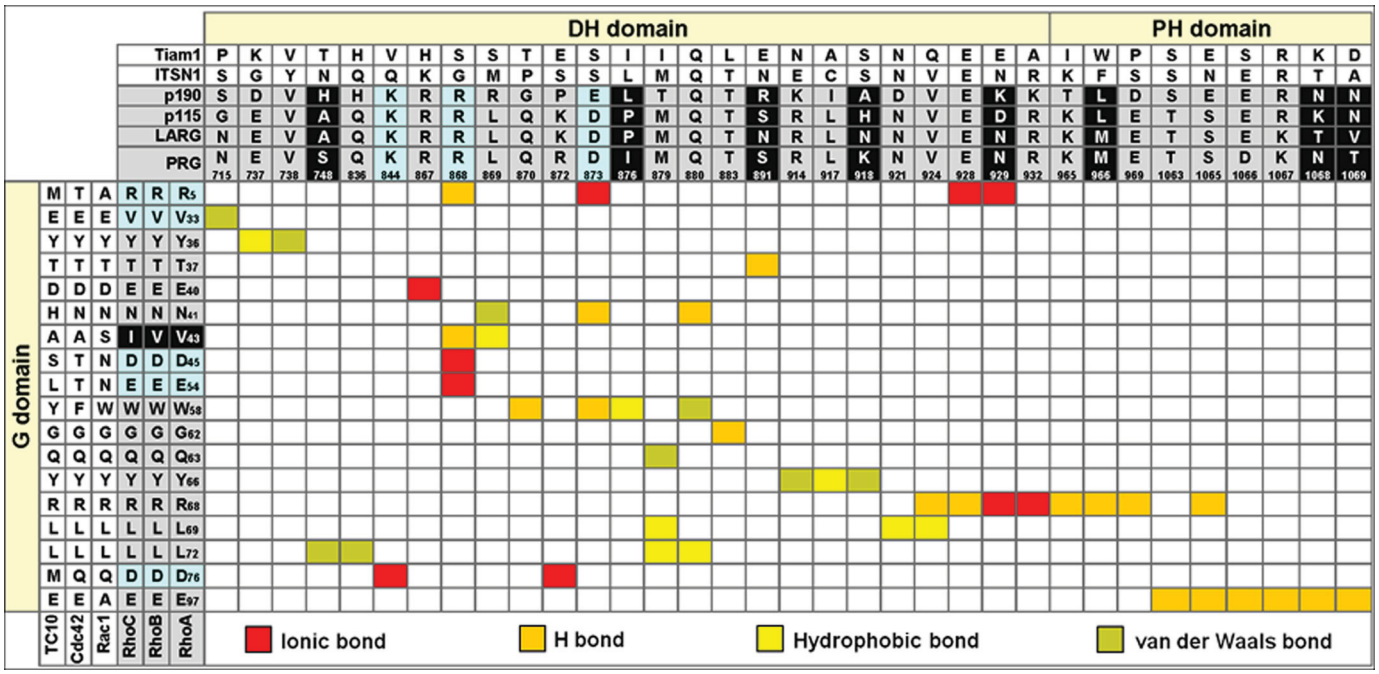

FIGURE 7. **Structure-based interaction sequence matrix illustrating specificity determining residues for RhoA interaction with its GEFs.** Based on the crystal structures of RhoA (G domain) in the complex with DH-PH of PRG (16) (Protein Data Bank code 1XCG) and of LARG (17) (Protein Data Bank code 1X86) the interacting residues (*colored background*; <4 Å in distance) were determined and aligned onto the DH-PH tandem and the G domain of RhoGTPases. Residues with a *light blue background* are conserved in Rho-specific GEFs and critical in determining the specificity of the RhoA/DH-PH interaction. Variable residues with a *black background* may be critical in determining the catalytic efficiency of Rho-specific GEFs.

domain by the RGS domain, we further analyzed the DH-catalyzed nucleotide dissociation in the presence of isolated RGS. The observed kinetic data (Fig. 6) revealed no interference of the RGS domain with the DH activity at all. These findings suggested that p115-mediated regulation of Rho activation is not mainly controlled directly by the RGS interaction with the DH domain but also by the linker region between RGS and DH domain. To test this hypothesis, we measured the DH-catalyzed mantGDP dissociation from RhoA in the presence of the linker and a mixture of the linker and the RGS domain, respectively. However, we did not detect any inhibition of the DH activity by these isolated domains even in the presence of a 100-fold excess of the RGS-Linker over the DH domain (Fig. 6). The same result was obtained when the p115 DH-PH tandem was used instead of the DH domain (data not shown). Taken together, our data support the previous reports that p115 underlies an autoinhibitory mechanism (27) that seems to be partially different compared with that of PRG, which also utilizes a cluster of acidic residues immediately upstream of the DH domain (37).

DISCUSSION

The cellular activity of small GTPases, such as RhoA, is determined by the nature of bound nucleotide and is strictly regulated. Activated GEFs, for example, accelerate the otherwise very slow exchange of GDP to GTP by several orders of magnitude. In this study, we used fluorescence spectroscopic methods to determine quantitatively (i) the specificity of four GEFs, p115, p190, PRG, and LARG on six RhoGTPases; (ii) their catalytic constants (k_{cat} and K_m) toward RhoA; (iii) the association of RhoGEF with GDP-bound fRhoA; and (iv) the influence of other domains, such as the PH and RGS domains, and an N-terminal segment on the DH capability in both binding fRhoA-GDP and catalyzing mantGDP dissociation from RhoA.

Rho Isoform Specificity of p115, p190, PRG, and LARG—We analyzed the activity of GEFs using mantGDP-bound RhoA isoforms and showed that isolated DH-PH domains represent the catalytic units of these GEFs. This is, in the first instance, consistent with previous results on p115 (25, 26, 28), p190 (39), PRG (16, 29, 62), and LARG (17, 30, 63) obtained by different kinds of assays. In addition, we clearly demonstrated that these GEFs do not exhibit any activity toward Rac1, Cdc42, and TC10 at all, suggesting their unique substrate specificity for the three isoforms RhoA, RhoB, and RhoC. GEFs of the Dbl family are mostly specific for one member or a subgroup of the Rho family GTPases (64). For example, hPEM-2/Collybistin, ITSN1, and Asef are specific for Cdc42 (44, 65, 66), whereas Tiam1 specifically activates Rac isoforms (41, 67, 68). On the other hand, there are GEFs with dual specificity, including Dbs as a GEF for RhoA and Cdc42 (69), Vav3 for RhoA and RhoG (70), and Trio for Rac1 and RhoG (71).

The high sequence conservation within both individual RhoGTPase members and various DH-PH domains (supplemental Fig. S2) raises the question of how the specificity of the RhoGEFs for the Rho isoforms is achieved. To address this important issue, we identified the contacting residues of the RhoA-PRG and RhoA-LARG complexes using the respective crystal structures (16, 17) and aligned them to various DH-PH tandems and to the G domains analyzed in this study (Fig. 7). Considering the interactions from the Rho side, five of 18 DH-PH-contacting residues (Arg^5, Val^{33}, Asp^{45}, Glu^{54}, and Asp^{76})

are identical in the RhoA, RhoB, and RhoC isoforms but variable in Cdc42, Rac1, and TC10 (Fig. 7, residues with *cyan background*).

Strikingly, these specificity-determining residues, except for Val^{33}, are not part of the switch regions (supplemental Fig. S2B) (4). Substitution of Val^{33} in RhoA (corresponding to Val^{33} in RhoB and RhoC, Glu^{31} in Rac1 and Cdc42, and Glu^{45} in TC10) by cysteine for fluorescence labeling did not show any significant influence on the GEF-catalyzed mantGDP dissociation either by LARG or p115 (data not shown). In addition, Val^{33} replacement by glutamate did not change the catalytic properties of LARG DH-PH (17). Val^{43} in RhoA and RhoB (Ile^{43} in RhoC) must also be included in this group of residues, which has been described previously to be critical for Rho recognition by GEFs (54). Its replacement by a Serine (equivalent to Ser^{41} in Rac1) resulted in a 25-fold reduction of the PRG DH-PH exchange activity, the most critical impairment among all tested mutants of RhoA (54).

Considering the interactions from the DH-PH side, three of 34 RhoA-contacting residues (supplemental Fig. S2A; N-terminal of the conserved region 3) are identical in Rho-specific PRG (Lys^{884}, Arg^{868}, and Asp^{873}), LARG, p115, and p190 but variable in GEFs specific for other members of the Rho family, *e.g.* the Cdc42-specific ITSN1 and the Rac1-specific Tiam1 (Fig. 7, residues with *cyan background*). These specificity-determining residues from both the Rho-specific GEFs and the Rho isoforms are strikingly linked together via ionic and H-bonds (Fig. 7, *red* and *orange fields*). The corresponding contacts are Arg^{5} with Arg^{868} and Asp^{871}, Asp^{45} and Glu^{54} with Arg^{868}, and Asp^{76} with Lys^{844}. Individual substitution of these residues of RhoA to Rac1 (R5A, D45N, E54N, and D76Q) or PRG to ITSN1 (R868G and D873S; Lys^{844} was not analyzed) has been shown to result in a drastic reduction of the PRG-catalyzed nucleotide dissociation up to 25-fold (54). The same study has shown that substitution of Rac1 and Cdc42 residues at four positions equivalent to RhoA residues (A3R, S41V, N45D, and N52E and T3R, A41V, T45D, and T52E, respectively) generates proteins whose nucleotide exchange can be significantly accelerated by PRG when compared with the wild type proteins.

Attributes of Catalytic Efficiency of RhoGEFs—A striking finding of this study is that PRG and LARG exhibited a GEF activity that was 2 orders of magnitude higher as compared with p115 and p190. This is particularly interesting because p115 belongs to the same subfamily of RGS-containing Dbl proteins as PRG and LARG (22, 72). An efficient catalytic activity of a GEF is dependent on at least two successive reactions: (i) association with RhoA-GDP and (ii) exchange of the bound GDP for GTP proceeding via a high affinity nucleotide-free GEF-RhoA reaction intermediate. To explain the catalytic efficiency of PRG and LARG *versus* p115, we first focused on the available structural data. From our structure-based interaction sequence matrix in which we inspected crystal structures of PRG (16) and LARG (17) in complex with RhoA, we selected nine variable residues contacting the nucleotide-free form of RhoA (Fig. 7, residues with *black background*). The three residues from the PH domain can be excluded because of the fact that this domain does not contribute to catalytic activity of LARG (see below). Within the other six residues, Ile^{876} substitution by proline (equivalent residue in LARG and p115) in PRG DH-PH has been shown in a comprehensive mutational study to generate a more efficient exchange factor (54), consistent with our observation that LARG exhibited a 4-fold higher activity than PRG. Two asparagines that are conserved in PRG (Asn^{715} and Asn^{928}) and LARG (Asn^{767} and Asn^{928}) appear to be critical for both DH-PH associations with RhoA and nucleotide exchange on RhoA (see below).

An obvious alternative explanation for the much lower efficiency of p115 GEF as compared with PRG and LARG is based on differences of the GEF association with the GDP-bound RhoA, which we found by developing a new method. Six of eight selected residues in LARG (Asn^{768}, Asp^{770}, Arg^{775}, Gly^{780}, Asn^{946}, and Lys^{949}) seem to play an important role in the association of LARG DH-PH with RhoA-GDP and for catalytic activity of LARG. Most of these residues are identical in PRG and in LARG, including Asn^{768}/Asn^{946} and Asn^{715}/Asn^{928} (Fig. 7, *black background*), and thus play a similar role in both the association and in the exchange reaction. The N-terminal segment of LARG DH-PH contains two short α-helices encompassing four of these residues (Asn^{768}, Asp^{770}, Arg^{775}, and Gly^{780}) from which only Asn^{768} contacts nucleotide-free RhoA (16). This suggests that the other three residues most likely contact RhoA in its nucleotide-bound form. We found that GEFs can equally recognize GDP- and GTP-bound RhoA, which also confirms the reversible character of the nucleotide exchange reaction. The molecular basis for the recognition of RhoA-GDP by GEFs seems to be mainly dependent on the β2-β3 regions (54, 66). There is only one crystal structure of a ternary complex (GTPase-GDP-GEF) known, which is the plant GTPase ROP4-GDP in complex with its GEF plant-specific ROP nucleotide exchanger (PRONE), that presents a common mechanism of catalyzed nucleotide exchange applicable to small GTPases in general (74). Rop4-contacting regions of PRONE are the P-loop, switch I, the β1 strand, part of switch II, and the end of the insert helix (supplemental Fig. S2B). This issue must be resolved structurally for the RhoA-related proteins.

Differential Roles of Tandem PH Domain—PH domains are best known for their ability to bind phosphoinositides with high affinity and specificity, although it is now clear that less than 10% of all PH domains share this property (75). Work with the Dbl family exchange factors consistently raises the question regarding the functional role of the tandem PH domain. Such an arrangement has been proposed to imply a crucial and unique functional interrelationship (5, 7). It has been shown that the PH domains of Trio, Dbs, and Dbl have a cooperative effect on the catalysis of the exchange reaction by the DH domain as its absence leads to a strong decrease in stimulation of the nucleotide dissociation (14, 76, 77). Our kinetic data of the exchange reaction imply that the PH domains contribute to the nucleotide exchange reaction mediated by the DH domain to different extents depending on the particular GEF. Compared with the activity of the isolated DH domain, the DH-PH tandem of PRG and p115 exhibited up to 5-fold enhanced exchange activities, respectively. This finding is supported by several previous studies. Wells *et al.* (28) reported that removal of the PH domain dramatically reduced the *in vitro* activity of p115 RhoGEF. The crucial role of the PH domain of PRG has been demonstrated previously for the catalysis of RhoA nucleotide exchange (16). Mutation of the PH binding residue of

RhoA was shown to affect strongly the catalytic function of PRG DH-PH protein (78). In addition to the interaction with nucleotide-free GTPase, PRG PH domain can also interact with GTP-bound RhoA, regulating cellular PRG activity (60). In contrast to PRG and p115, the influence of the PH domain on the activity of the DH domain of p190 and LARG was rather insignificant and very similar to the Cdc42-specific Asef (44). These data indicate that the DH domains of p190 and LARG represent the entire catalytic machinery to accomplish Rho activation and that their PH domains do not contribute to Rho activation.

One possible role of the PH domain on the activity of the DH domain might be its direct interaction with the GTPase (16). For example, the x-ray structure of Dbl in complex with Cdc42 (14, 15) and structures of Dbs (13), PRG (16, 54, 55, 78), and LARG (17) in complex with the nucleotide-free RhoA revealed that the PH domains of these GEFs directly contact switch II and the α3 helix of the respective GTPase. On the other hand, no direct interaction between the PH domain and GTPase was observed in complex structures of Rac1-Tiam1 (20), Rac1-Trio (76), Cdc42-ITSN1 (14), and Cdc42-Collybistin (21) and in the ternary complex $G\alpha_q$-$p63^{RhoGEF}$-RhoA (79). Interestingly, despite no interaction of the PH domain of Trio in the complex structure with Rac1, its absence caused a 4-fold decrease in exchange activity (76). A nearly opposite scenario is observed for LARG that contacts the GTPase with its PH domain (17) in the same manner as shown for PRG (16). Although PRG PH clearly contributes to kinetics of nucleotide exchange, LARG PH is dispensable for the DH activity *in vitro*. Interestingly, a conserved hydrophobic patch of the LARG PH domain has been reported recently to be critical for RhoA activation in cells (80). It has been suggested that the LARG PH domain is involved in regulatory interactions with other proteins near the membrane surface. It is assumed that in cells GEFs are directionally translocated to the plasma membrane in response to extracellular signals (12) where they are localized to posttranslationally modified small GTPases. In an *in vitro* liposome reconstitution, Robbe *et al.* (81) have shown that Tiam1 DH-PH, which specifically accelerates nucleotide exchange of the Rac isoforms (41, 42), activates prenylated Rac1 much more efficiently in the presence of liposomes. A model for PH domain-assisted nucleotide exchange has been proposed for Dbs and also for other GEFs such as Dbl and Trio. Herein the PH domain serves multiple roles in signaling events anchoring GEFs to the membrane (via phosphoinositides), directing them toward their interacting GTPases, which are already attached to the membrane (14, 22, 77, 82–85).

p115 Autoinhibition—The RGS-containing RhoGEFs, including p115, PRG, and LARG, represent a distinct family of guanine nucleotide exchange factors for RhoA that are regulated by the $G\alpha_{12/13}$ proteins. Experimental evidence indicates that the complex architecture of these RhoGEFs provides the structural basis for regulatory mechanisms mediated by protein-protein interactions (26, 86). Association of the RGS domain of p115 with $G\alpha_{12/13}$ proteins was shown to partially activate its GEF activity toward RhoA, suggesting that the N terminus of p115 may contribute to autoinhibition of the DH-PH activity (26). Accordingly, we could demonstrate that a large protein fragment consisting of RGS-Linker-DH (residues 40–645) indeed exhibited significantly reduced GEF activity. This suggests that regions upstream of the DH domain may interact in intramolecular fashion with the DH domain and mask it from binding to RhoA-GDP. Such an apparent autoinhibition of p115 DH activity could not be verified when its N-terminal regions, including RGS, Linker, and RGS-Linker, were separately mixed with the DH domain. Even at very high concentrations of the respective proteins (100-fold above the DH domain), we could not detect any *trans* inhibition of the DH activity by the N-terminal regions. The most recent structural analysis of p115 has shown that an N-terminal extension of the DH domain appears to play a critical role in p115 autoinhibition (60). Similarly, structural and biochemical analysis of various PRG proteins reported by Zheng *et al.* (37) has provided new insight into the molecular nature of such an intramolecular interaction. PRG utilizes an electrostatic patch immediately upstream of the DH domain and contributes in part to an autoinhibitory mechanism that appears to require additional regions of the full-length protein, including the RGS domain.

In summary, our data strongly support the conclusion that the DH domain of the RhoGEF itself determines the specificity for binding RhoGTPases and represents very efficient catalytic machinery for the nucleotide exchange in a cell-free and membrane-free system. In cells, however, a set of additional domains and interactions are required for the shuttling, localization, and activation of the GEFs (11, 81, 73). Complex formation of GEFs with receptors (*e.g.* semaphorin receptors/plexins) and G-proteins (*e.g.* $G\alpha_{12/13}$) at the membrane are required for the functional activation and the regulation of cellular processes, including adhesion, contraction, and motility.

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Supplementary Materials

Mechanistic Insights into Specificity, Activity and Regulatory Elements of the RGS-containing Rho-specific Guanine Nucleotide Exchange Factors p115, PDZ-RhoGEF (PRG) and Leukemia-associated RhoGEF (LARG)

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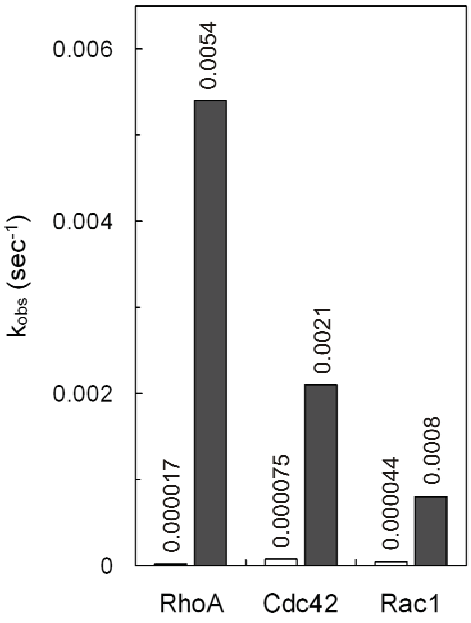
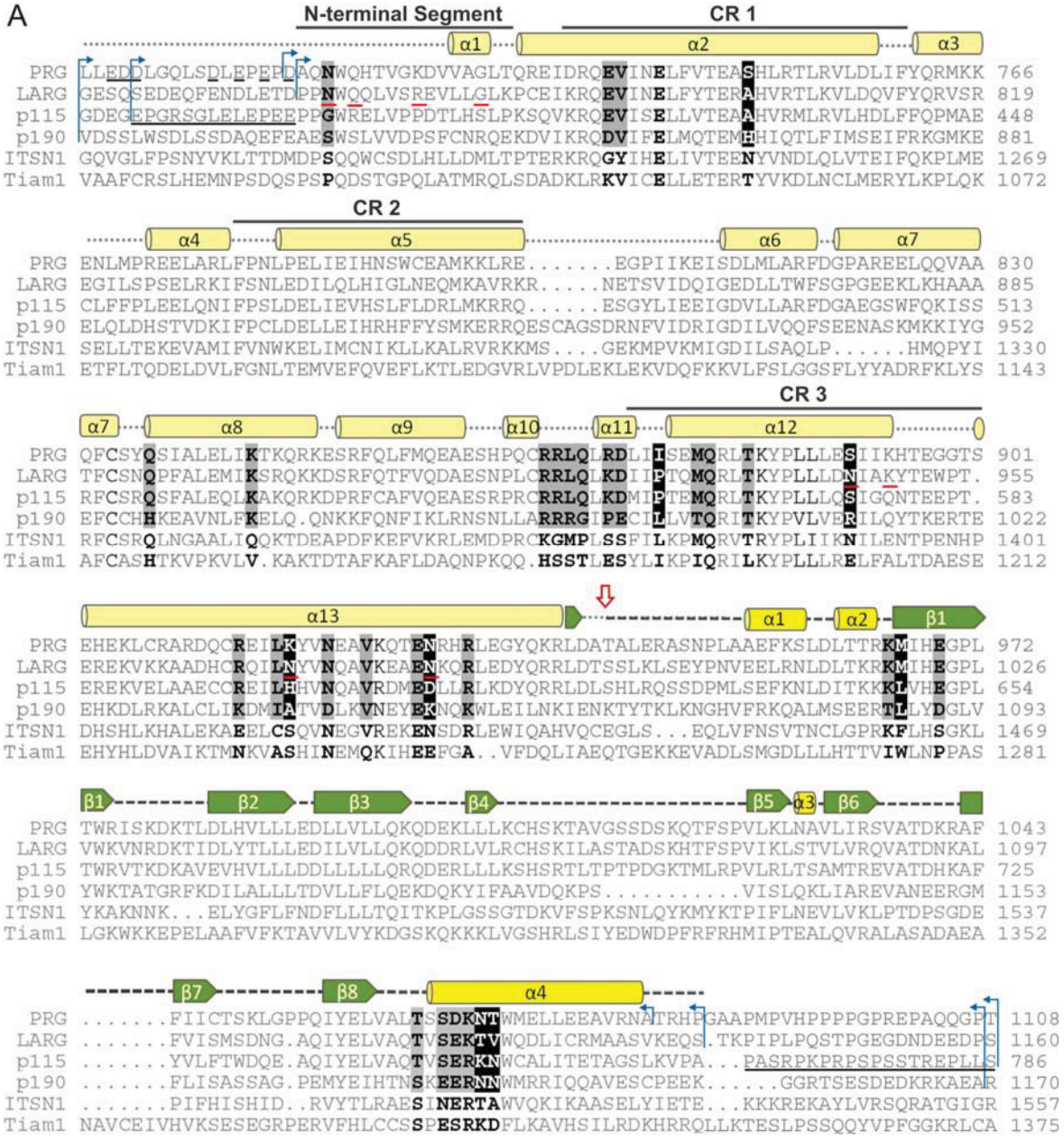


Fig. S1 Nucleotide exchange activity of RhoGEFs. The DH-PH domain of p115, Asef and Tiam1 catalyzes specifically the mantGDP exchange reaction of RhoA, Cdc42 and Rac1, respectively (conditions are decribed in Fig. 2; white bars: no GEF; black bars: GEF added).



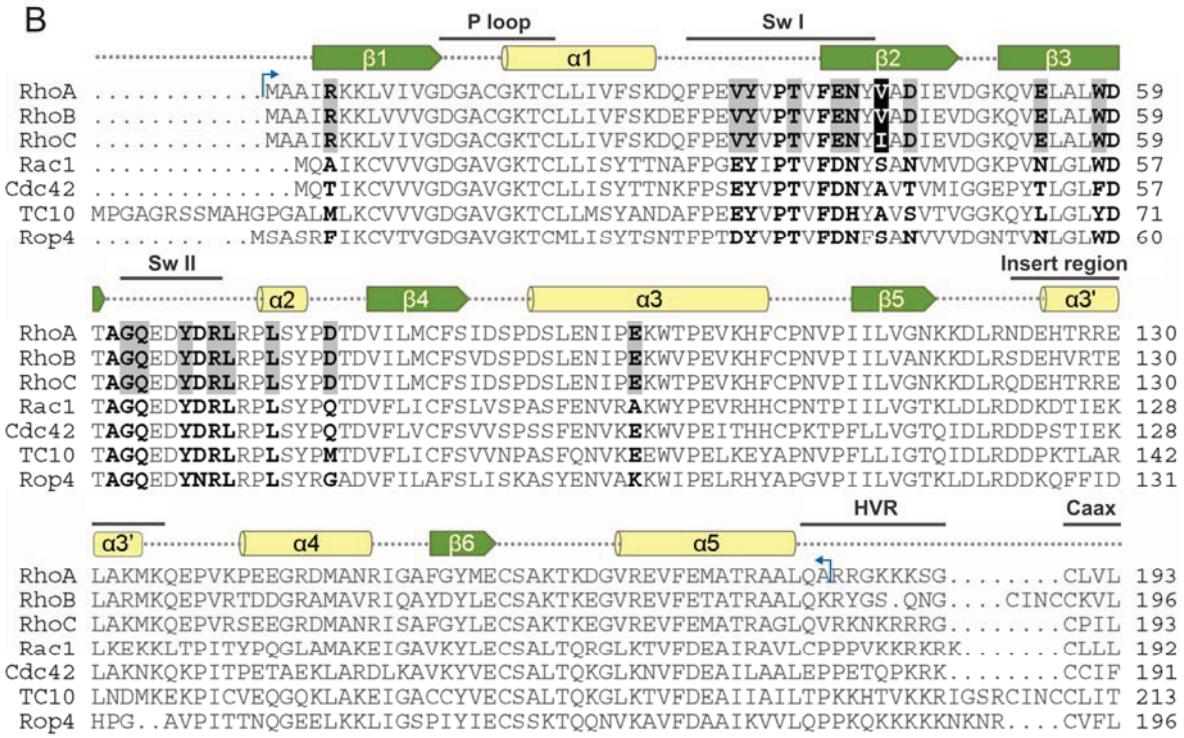


Fig. S2 **Multiple sequence alignment of DH-PH-containing GEFs (A) and Rho GTPases (B).** The DH-PH domains of Tiam1 (RacGEF), ITSN1 (Cdc42GEF) and the Rho specific GEFs p190, p115, LARG and PRG along with full length TC10, Cdc42, Rac1, RhoC, RhoB and RhoA are used to highlight and to discuss the specificity determining residues based on the crystal structures of RhoA in the complex with DH-PH of PRG (16)) (PDB ID 1XCG) and of LARG (17)) (PDB ID 1X86). All bold residues (**X**, **X**, **X**) are involved in the RhoA/DH-PH interaction. Black residues in bold with a grey background (**X**) are conserved and important in determining the specificity of the RhoA/DH-PH interaction. White residues in bold and black background (**X**) are variable and involved in assigning specificity. **X** and **X** are selected in Fig. 7 to discuss the biochemical data in this study. The conserved signatures of the Rho GTPases are highlighted: P loop, switch I, switch II, hypervariable region (HVR) and the prenylation site (CaaX). Conserved regions within the DH domain (CR1, CR2, CR3) and the N-terminal segments are shown. Black lines indicate the termini of p115 DH-PH that are truncated in DH-PHc and DH-PHcn of p115. The polypeptide backbone is shown as a dashed line and the secondary structure elements (alpha-helices and beta sheets) are illustrated based on the crystal structures of RhoA in the complex with the DH-PH of PRG (16)). An arrow (⇩) indicate the C-terminus of the DH and the N-terminus of the PH domains and blue arrows (↱) indicate the respective N-terminal and C-terminal ends of the proteins used in this study. Amino acids underlined in red in LARG may be responsible for the highly efficient exchange activity of LARG and PRG versus p115 and p190. Amino acids underlined in black at the N-terminus of PRG DH domain has been shown to have inhibitory effects on the PRG exchange activity (37).

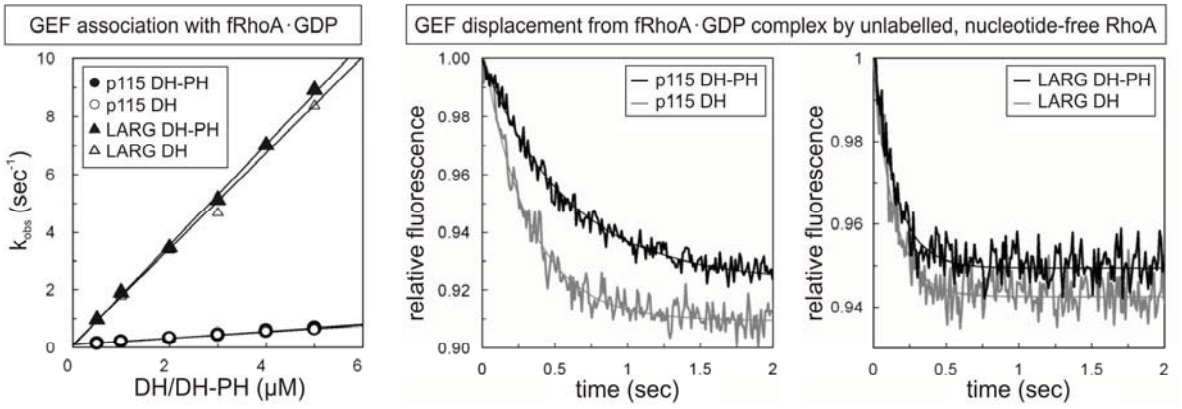


Fig. S3 Real-time monitoring of the RhoGEF interactions with the fluorescently labelled GDP-bound RhoA (fRhoA). Kinetics of the association between fRhoA and the DH and DH-PH domains of LARG and p115, respectively, at the left panel clearly revealed differential binding properties of the two RhoGEFs. Observed rate constants (k_{obs}) of the association curves obtained at increasing DH and DH-PH concentrations were calculated by single exponential fitting. The association rate constants (k_{on}) of fRhoA·GDP-binding to the DH and DH-PH proteins were calculated from the linear regression of the k_{obs} values plotted against the concentrations of the DH-PH (closed symbols) and the DH (open symbols) domains of LARG (triangle) and p115 (circles). The DH-PH (10 μM) dissociation from the fRhoA·GDP complex (middle and right panels) was measured in a displacement experiment in the presence of excess amounts of unlabelled, nucleotide-free RhoA (20 μM). The dissociation rate constant (k_{off}) was determined by an exponential fit of the data. The dissociation constant (K_d) was calculated from the kinetic parameters of dissociation and association reactions by the equation: $K_d = k_{off} / k_{on}$.

Supplementary Information

Deciphering the molecular and functional basis of Dbl family proteins: A novel systematic approach towards classification of selective activation of the Rho family proteins

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Table S1 Published 3D structure of the Rho and Dbl proteins, and their complexes (page 1)

Rho protein structures				
No.	**Protein**	**PDB ID**	**Resolution (Å)**	**Reference**
1	RhoA(F25N)	1FTN	2.10	(1)
2	RhoA	1A2B	2.40	(2)
3	RhoA	1DPF	2.00	(3)
4	RhoA(Q63L,F25N)	1KMQ	1.55	(4)
5	RhoB	2FV8	1.90	to be published
6	RhoC	2GCN	1.85	(5)
7	RhoC	2GCO	1.40	(5)
8	RhoC	2GCP	2.15	(5)
9	Cdc42Hs	1AN0	2.80	to be published
10	Cdc42(G12V)	1A4R	2.50	(6)
11	Cdc42Hs	1AJE	NMR	(7)
12	Cdc42(T35A)	2KB0	NMR	to be published
13	Cdc42Hs(F28L)	2ASE	NMR	(8)
14	Rac1	2P2L	1.90	(9)
15	Rac1b	1RYH	1.75	(10)
16	Rac1b	1RYF	1.75	(10)
17	Rac2(G12V)	2W2T	1.95	(11)
18	Rac2(G12V)	2W2V	2.00	(11)
19	Rac3	2IC5	1.90	to be published
20	Rac3	2G0N	1.90	to be published
21	Rac3	2C2H	1.85	to be published
22	RhoD	2J1L	2.50	to be published
23	TC10	2ATX	2.65	(12)
24	Wrch1	2Q3H	1.73	to be published
25	Rnd1	2CLS	2.31	to be published
26	Rnd3	1M7B	2.00	(10)
27	Rnd3	1GWN	2.10	(13)
Dbl protein structures				
No.	**Protein**	**PDB ID**	**Resolution (Å)**	**Reference**
1	ASEF	2DX1	2.36	(14)
2	ASEF	2PZ1	2.25	(15)
3	βPix DH	1BY1	NMR	(16)
4	Dbs DH-PH	1RJ2	3.00	(17)
5	FGD5 DH-PH	3MPX	2.80	to be published
6	ITSN2	3GF9	2.50	to be published
7	ITSN1L SH3E-DH	3JV3	2.40	(18)
8	Kalirin DH1	2KR9	NMR	to be published
9	LARG DH-PH	1TXD	2.13	(19)
10	Net1	3EO2	2.60	to be published
11	p115 DH-PH	3ODO	2.90	(20)
12	p115 DH-PH (R399E)	3P6A	2.50	(20)
13	p115 ΔN Linker-DH-PH	3ODX	3.20	(20)
14	p115 Linker-DH-PH	3ODW	3.20	(20)
15	Sos1 DH-PH-cat	1XD4	3.64	(21)
16	TrioN DH-PH	1NTY	1.70	(22)
17	VAV1 CH-DH-PH-C1	3KY9	2.73	(23)
18	VAV DH	1F5X	NMR	(16)
19	XPLN DH-PH	2Z0Q	1.79	to be published

Table S1 Published 3D structure of the Rho and Dbl proteins, and their complexes (page 2)

Dbl-Rho protein complexes				
No.	**Protein**	**PDB ID**	**Resolution (Å)**	**Reference**
1	RhoA·Dbs·	1LB1	2.81	(24)
2	RhoA·LARG	1X86	3.22	(19)
3	RhoA·PRG	1XCG	2.50	(25)
4	RhoA·PRG	3T06	2.84	(26)
5	RhoA·GTPγS·PRG	3KZ1	2.70	(27)
6	RhoA·p63	2RGN	3.50	(28)
7	Cdc42·Collybistin II	2DFK	2.15	(29)
8	Cdc42·Dbs	1KZ7	2.40	(30)
9	Cdc42 (Y889F)·Dbs	1KZG	2.60	(30)
10	Cdc42·ITSN1	1KI1	2.30	(24)
11	Rac1·VAV1	2VRW	1.85	(31)
12	Rac1·VAV1	3BJI	2.60	(32)
13	Rac1·Tiam1	1FOE	2.80	(33)
14	Rac1·TrioN	2NZ8	2.00	(34)

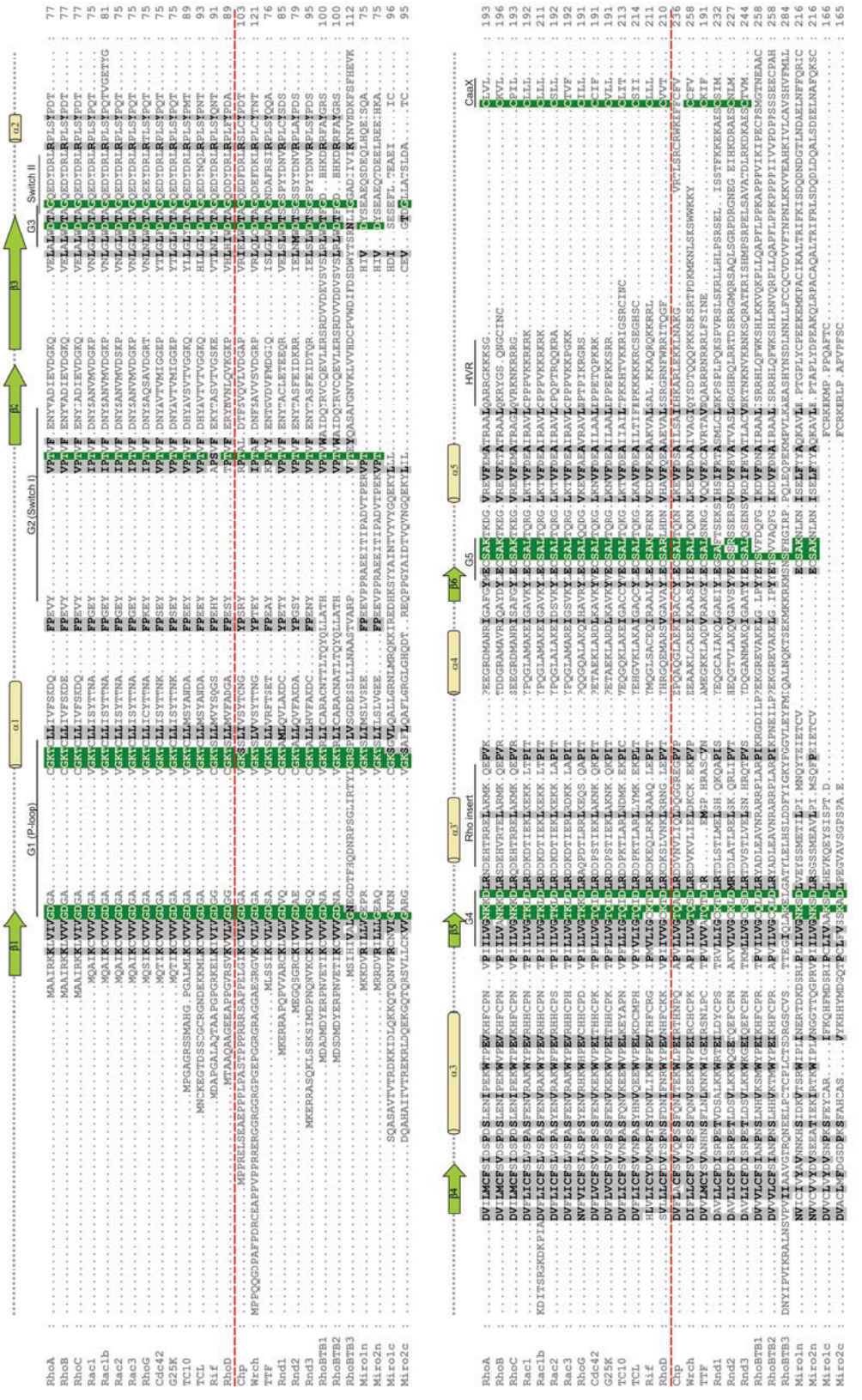

Figure S1 Multiple sequence alignment of Rho protein family. The Amino acid sequences of 25 Rho family proteins were aligned using ClustalW program. The alignment was edited by GeneDoc and adjusted manually by

eye. The conserved signatures of the Rho proteins are represented as G1 (or P loop for phosphate binding and magnesium ion coordination), G2 (or switch I for magnesium ion coordination and γ-phosphate binding), G3 (or switch II for γ-phosphate binding containing the catalytic glutamine), G4 (major determinant of guanine base binding specificity) and G5 box (for guanine base binding). Conserved residues in G1-G5 signature motifs are highlighted as (X) and residues that are 80% conserved are highlighted as (X). Secondary structural elements are presented on the top of an alignment, helices as cylinder and beta sheets by an arrow. Proteins below the dash line (---) are not included in this study. Large C-terminal extensions for RhoBTB proteins and TTF are not shown. Miro and RhoBTB proteins show high variability within the base and ribose binding motifs. Miro1 and Miro2 contain two G domains (termed as Miro1n, Miro1c, Miro2n and Miro2c, respectively). Miro1n and Miro2n share certain similarity to typical Rho proteins, lacking both an insert helix and a C-terminal CaaX-motif.

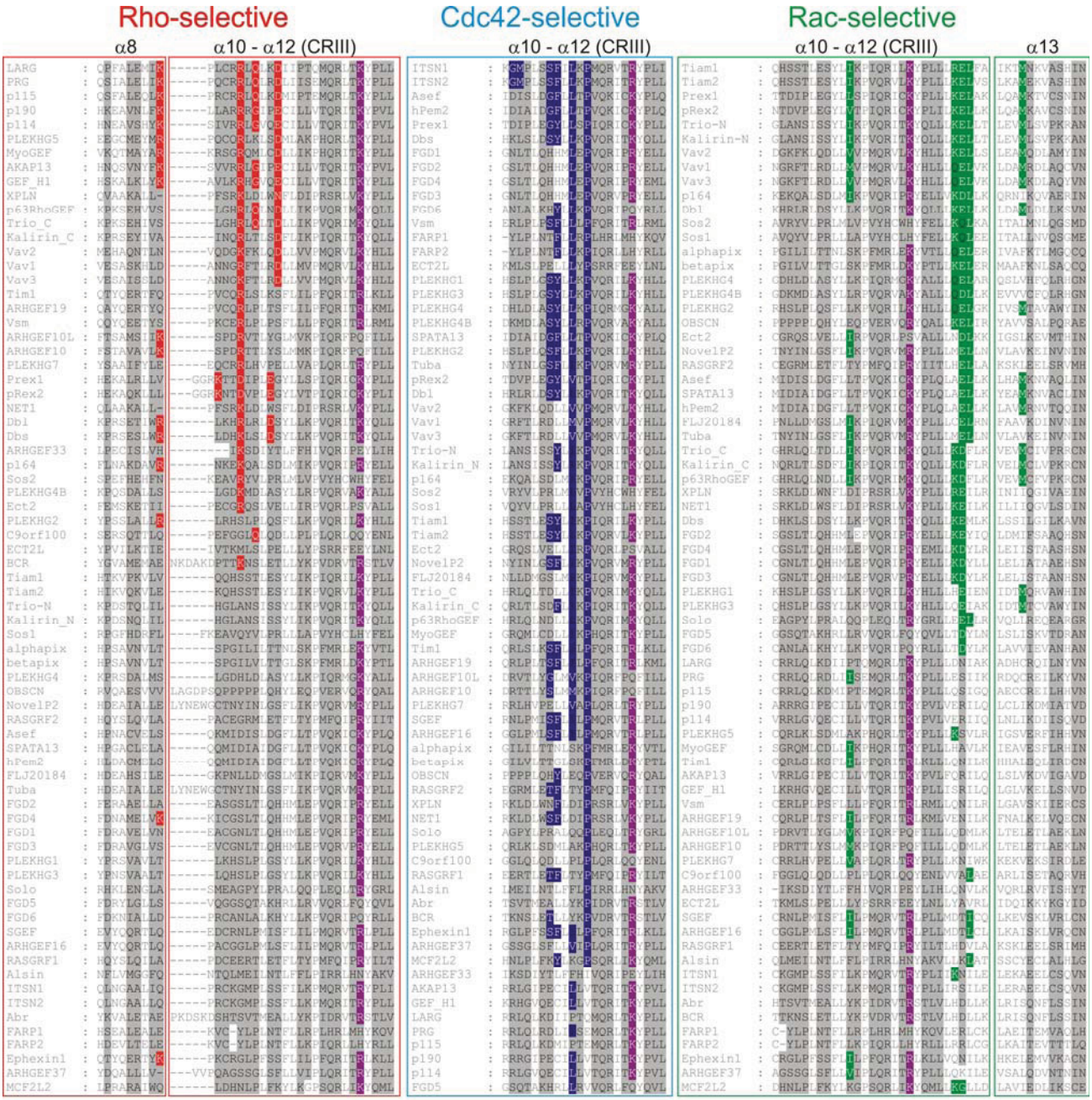

Figure S2 Multiple sequence alignment of Dbl-homology (DH) domain of GEFs. Comprehensive analysis of the amino acid sequences of all human Dbl proteins along with all biochemical and structural data of this and other studies resulted in the determination of regions and residues essential for selective interaction with Rho-, Cdc42- and Rac-selective proteins. For the alignment of Dbl family proteins, we have used several criteria to search and predict Dbl family proteins in human genome. The representative amino acid sequences were obtained from National center of Biotechnology Information (NCBI) database, Pfam databse and SwissProt database. Their corresponding DH and PH domain were predicted from SMART database. Apart from above databases, “RhoGEFs” and “ARHGEFs” as keywords were used to achieve more proteins. All sequences were

then checked to remove redundancy. As a result we have found 74 Dbl family proteins containing DH domain as signature motif. Sequences for each Dbl family were aligned by using MUSCLE (35) with default multiple alignment parameters. The alignment was later edited by GeneDoc and adjusted by eye. The sequence conservation within, but not between, subfamilies appears to be due to conservation of function within the subfamilies. The regions conserved within subfamilies may be important in providing specific functions to each subfamily. In this case, the functions of the uncharacterized proteins in the Dbl family can be predicted by comparison with other members of the same subfamily. For assigning the specificity, conserved residues in all 74 Dbl proteins were excluded. The Dbl proteins were then grouped on the basis of presences specificity determining residues in to Rho-, Cdc42- and Rac1-specific subfamilies. Residues responsible for RhoA (red), Cdc42 (blue), Rac1 (green) specificity (19,24,30,33,36) were searched for their presence in other Dbl proteins. As shown in this Figure, the conserved region III (CRIII) is critical for the recognition and substrate selectivity as reported previously for Tiam1 and PRG (37,38). For better orientation, we marked a highly conserved lysine in magenta. In addition, those Dbl proteins having combination of residues were divided in Rho-Cdc42-, Cdc42-Rac1-specific Dbl proteins. Those proteins, which contained all specificity determining residues, were classified as Rho-Rac-Cdc42-specific Dbl proteins. Dbl proteins, which did not contain any of these residues, were not assigned in any category.

Supplementary References

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Supplementary Information

Deciphering the molecular and functional basis of Dbl family proteins: A novel systematic approach towards classification of selective activation of the Rho family proteins

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Table S1 Published 3D structure of the Rho and Dbl proteins, and their complexes (page 1)

Rho protein structures				
No.	**Protein**	**PDB ID**	**Resolution (Å)**	**Reference**
1	RhoA(F25N)	1FTN	2.10	(1)
2	RhoA	1A2B	2.40	(2)
3	RhoA	1DPF	2.00	(3)
4	RhoA(Q63L,F25N)	1KMQ	1.55	(4)
5	RhoB	2FV8	1.90	to be published
6	RhoC	2GCN	1.85	(5)
7	RhoC	2GCO	1.40	(5)
8	RhoC	2GCP	2.15	(5)
9	Cdc42Hs	1AN0	2.80	to be published
10	Cdc42(G12V)	1A4R	2.50	(6)
11	Cdc42Hs	1AJE	NMR	(7)
12	Cdc42(T35A)	2KB0	NMR	to be published
13	Cdc42Hs(F28L)	2ASE	NMR	(8)
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15	Rac1b	1RYH	1.75	(10)
16	Rac1b	1RYF	1.75	(10)
17	Rac2(G12V)	2W2T	1.95	(11)
18	Rac2(G12V)	2W2V	2.00	(11)
19	Rac3	2IC5	1.90	to be published
20	Rac3	2G0N	1.90	to be published
21	Rac3	2C2H	1.85	to be published
22	RhoD	2J1L	2.50	to be published
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12	p115 DH-PH (R399E)	3P6A	2.50	(20)
13	p115 ΔN Linker-DH-PH	3ODX	3.20	(20)
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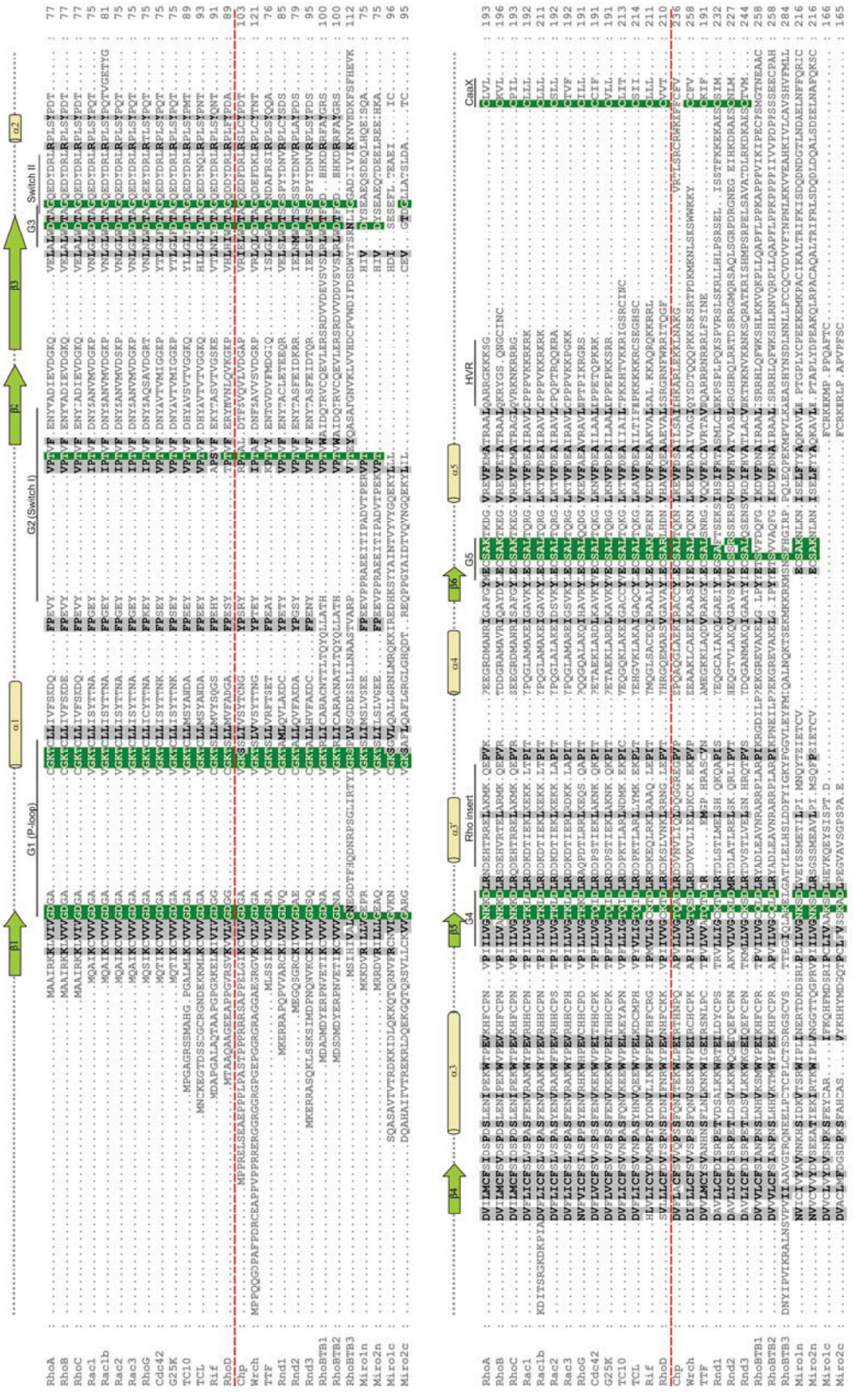

Figure S1 Multiple sequence alignment of Rho protein family. The Amino acid sequences of 25 Rho family proteins were aligned using ClustalW program. The alignment was edited by GeneDoc and adjusted manually by

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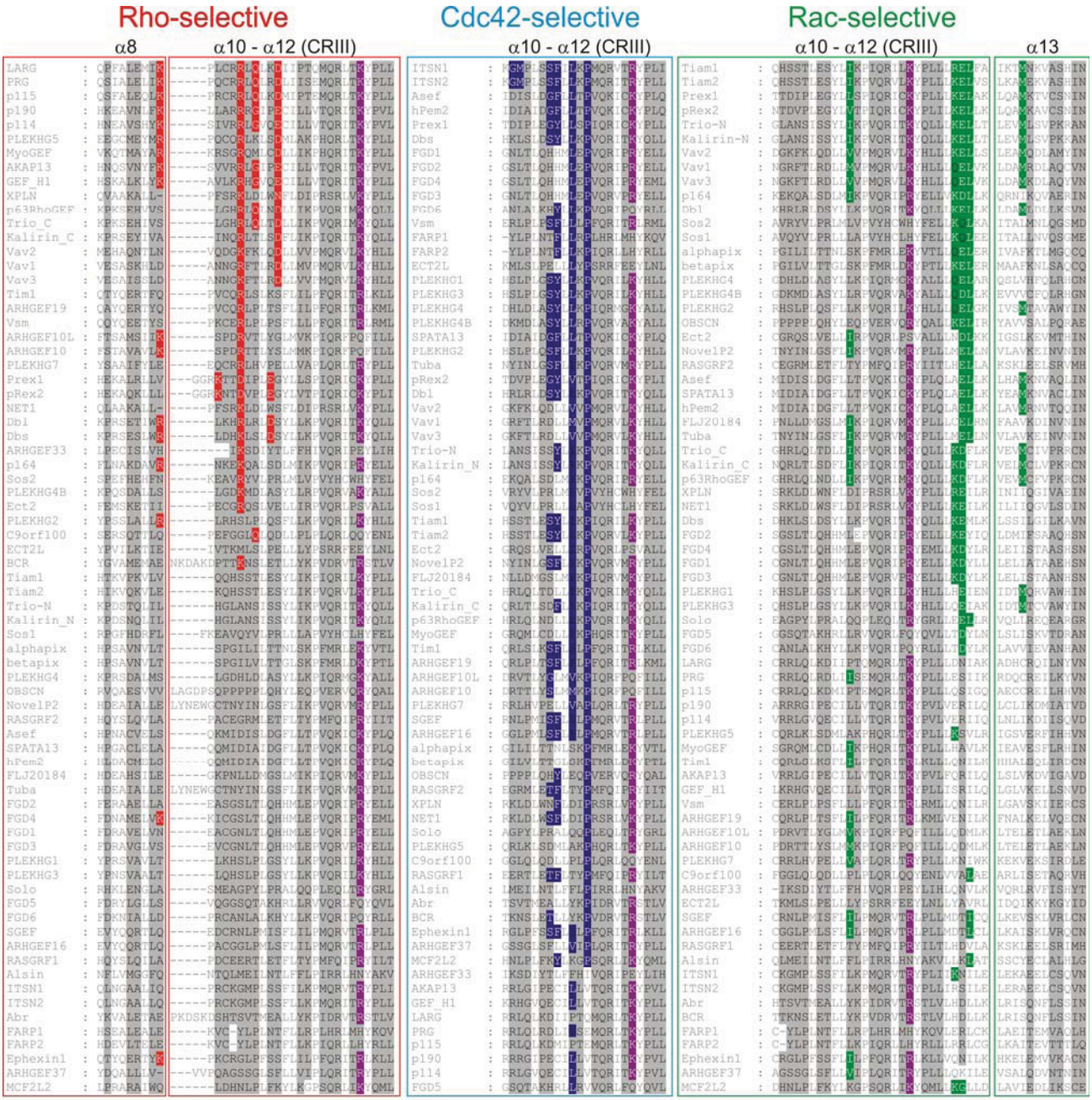

Figure S2 Multiple sequence alignment of Dbl-homology (DH) domain of GEFs. Comprehensive analysis of the amino acid sequences of all human Dbl proteins along with all biochemical and structural data of this and other studies resulted in the determination of regions and residues essential for selective interaction with Rho-, Cdc42- and Rac-selective proteins. For the alignment of Dbl family proteins, we have used several criteria to search and predict Dbl family proteins in human genome. The representative amino acid sequences were obtained from National center of Biotechnology Information (NCBI) database, Pfam databse and SwissProt database. Their corresponding DH and PH domain were predicted from SMART database. Apart from above databases, “RhoGEFs” and “ARHGEFs” as keywords were used to achieve more proteins. All sequences were

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Supplementary References

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Research Article

The tumor suppressor protein DLC1 is regulated by PKD-mediated GAP domain phosphorylation

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ABSTRACT

Deleted in liver cancer 1 (DLC1) is a tumor suppressor protein that is frequently downregulated in various tumor types. DLC1 contains a Rho GTPase activating protein (GAP) domain that appears to be required for its tumor suppressive functions. Little is known about the molecular mechanisms that regulate DLC1. By mass spectrometry we have mapped a novel phosphorylation site within the DLC1 GAP domain on serine 807. Using a phospho-S807-specific antibody, our results identify protein kinase D (PKD) to phosphorylate this site in DLC1 in intact cells. Although phosphorylation on serine 807 did not directly impact on *in vitro* GAP activity, a DLC1 serine-to-alanine exchange mutant inhibited colony formation more potently than the wild type protein. Our results thus show that PKD-mediated phosphorylation of DLC1 on serine 807 negatively regulates DLC1 cellular function.

Introduction

The DLC1 gene was first isolated as a candidate tumor suppressor gene in primary human hepatocellular carcinoma and loss of expression has subsequently been shown in various other tumor types, ranging from colon, breast to prostate [1]. Comparison of different breast cancer sublines by transcriptional profiling revealed that DLC1 expression is linked to their metastatic potential, with downregulation favoring the formation of pulmonary metastases in athymic mice [2]. Recently, proof for an *in vivo* tumor suppressor function of DLC1 was provided by a study in which DLC1 loss was shown to cooperate with myc overexpression in p53 null cells to promote liver tumorigenesis in mice [3].

DLC1 is a GTPase activating protein (GAP) that exhibits *in vitro* GAP activity for RhoA, RhoB and RhoC, and to a lesser extent Cdc42 [4]. The Rho family of GTPases are important regulators of diverse

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Abbreviations: DLC1, deleted in liver cancer 1; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; PDBu, phorbol-12,13-dibutyrate; PKD, protein kinase D
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biological responses, including actin cytoskeletal rearrangements, gene transcription, cell cycle regulation, apoptosis and membrane trafficking [5]. Rho proteins cycle between a GTP-bound active state to interact with effector proteins, modulating their activity and localization, and an inactive GDP-bound state. This activation of Rho proteins is controlled by the action of the guanine nucleotide exchange factors (GEFs), which promote the release of bound GDP and facilitate GTP binding, and the GAP proteins, which increase the intrinsic GTPase activity of Rho GTPases to accelerate the return to the inactive state. In accordance with its function as a regulator of Rho signaling, stable expression of human DLC1 in a hepatocellular carcinoma line suppressed Rho-driven processes, such as cell motility and invasiveness [6], whereas DLC1 downregulation by RNA interference increased directed cell migration of breast cancer cells via the Rho effector protein Dia1 [7].

Interestingly, no constitutively activating Rho protein mutants have been identified in human tumors. Rho proteins are rather found to be overexpressed; alternatively, aberrant Rho activation can be achieved by loss of negative regulators, the RhoGAPs. DLC1 is frequently downregulated due to deletion or promoter hypermethylation. Those tumors that still express DLC1 may have developed other means to inactivate its regulatory function within the cell. For example, by DNA sequencing of prostate and colon cancer samples, two somatic mutations (T301K and S308I) in DLC1 were identified that impair RhoGAP activity and consequently the ability to inhibit cancer cell proliferation [8]. Phosphorylation of the rat DLC1 protein in response to insulin has been reported, however, the functional consequences of this phosphorylation are unknown [9]. We previously identified PKD to phosphorylate DLC1 on serines 327 and 431, thereby creating recognition motifs for the phosphoserine-/phosphothreonine-binding 14-3-3 adaptor protein family [10]. 14-3-3 binding inhibited cellular DLC1 GAP activity, most likely by sequestration of DLC1 in the cytoplasm. Accordingly, DLC1-mediated inhibition of cell spreading was alleviated upon co-expression of 14-3-3 proteins and a DLC1 mutant defective in 14-3-3 binding (S327/431A) inhibited cell proliferation more potently than the wild type protein [10]. We now provide evidence that PKD phosphorylates an additional site in DLC1, namely serine 807 within the GAP domain, adding another layer of complexity to PKD-mediated negative regulation of the DLC1 tumor suppressor protein.

Materials and methods

Antibodies and reagents

Antibodies used were: monoclonal mouse anti-GFP mAb (Roche Biosciences, Germany), monoclonal mouse anti-Flag and anti-α-tubulin mAbs (Sigma-Aldrich, Germany), monoclonal mouse anti-DLC1 (BD Biosciences, Germany), polyclonal rabbit anti-PKD (C20; Santa Cruz). PKD-(P)S910 antiserum was described previously [11]. The DLC1-p807 antiserum was raised by immunizing rabbits with NH2-CREN(pS)SPR-CONH2 peptide (Pineda-Abservice, Germany). HRP-labeled anti-mouse and anti-rabbit IgGs were from GE Healthcare, Germany. Gö6976, Gö6983, phorbol-12,13-dibutyrate (PDBu) and doxycycline were from Merck Biosciences (Germany), staurosporine was from Alexis (Germany).

DNA cloning

pEGFPC1-DLC1 wild type and K714E, pEFrPuroFlag-DLC1, pEGFPN1-PKD1, pEGFPN1-PKD2, pcDNA3-PKD1, pcDNA3-PKD2, and pCR-Met-Flag-PKD3 were described previously [7,10]. The DLC1 GAP domain (amino acids 609–878) was subcloned by PCR into the pGEX4T1-NTEV vector using BamHI and Not1 restriction sites. Expression vectors encoding RhoA-G14V and RhoA-Q63L were kindly provided by John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). DLC1 point mutants were generated by QuikChange site-directed PCR mutagenesis according to the manufacturer's instructions (Stratagene, Germany). The forward primers used were: DLC1-S807A-F 5′-CTG AAG AGA GAG AAT GCC TCT CCC AGG GTA ATG-3′, DLC1-S807D-F 5′-CCC TGA AGA GAG AGA ATG ACT CTC CCA GGG TAA TGC-3′, and DLC1-S807E-F 5′-CAC CCT GAA GAG AGA GAA TGA GTC TCC CAG GGT AAT GC-3′. All amplified cDNAs were verified by sequencing. Oligonucleotides were purchased from MWG Biotech (Germany).

Cell culture and transfection

HEK293T and MCF7 cells were cultured in RPMI media (Invitrogen, Germany) supplemented with 10% FCS (PAA, Germany) in a humidified atmosphere of 5% CO_2 at 37 °C. Flp-In T-Rex HEK293 cells inducibly expressing GFP-DLC1 [10] were grown in RPMI containing 10% FCS, 50 μg/ml hygromycin and 15 μg/ml blasticidin. HEK293T cells were transfected using TransIt reagent (Mirus Bio LLC, Madison, USA), MCF7 cells were transfected using Lipofectamine 2000 (Invitrogen, Germany) or by nucleofection (Kit V, program P020; Amaxa, Germany).

Immunoprecipitations and Western blotting

Whole cell extracts (WCE) were obtained by solubilizing cells in TEB buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete protease inhibitors (Roche)]. Lysates were clarified by centrifugation at 16,000*g* for 10 min. For immunoprecipitations, equal amounts of protein were incubated with specific antibodies for 4 h on ice. Immune complexes were collected with protein G-Sepharose (GE Healthcare) and washed 3× with TEB. Precipitated proteins were released by boiling in sample buffer, subjected to SDS-PAGE and blotted onto PVDF membranes (Roth). After blocking with 0.5% blocking reagent (Roche) in PBS containing 0.1% Tween 20, filters were probed with specific antibodies. Proteins were visualized with HRP-coupled secondary antibody using the ECL detection system (Pierce).

MALDI-TOF mass spectrometry

HEK293T cells transiently expressing Flag-tagged DLC1 (5×10 cm dishes) were lysed in TEB and DLC1 was immunoprecipitated with anti-Flag M2 agarose (Sigma-Aldrich). The beads were washed with TEB and PBS and the protein was then eluted with 0.1 M glycine, pH 2.5 and neutralised with 1/10 volume 1 M Tris, pH 8. The eluted protein was concentrated by ultrafiltration, subjected to SDS-PAGE and stained with Colloidal Coomassie Blue (Roth). The band corresponding to DLC1 was excised and digested with trypsin as described [12]. Phosphopeptide enrichment was carried as described previously [13] with alterations to improve

phosphopeptide binding and protocol sensitivity [14]. MALDI-TOF mass spectra were acquired using a Bruker ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) operating in reflectron mode. Precursor ions of interest were chosen for MS/MS analysis. MALDI-TOF/TOF was performed in the MS/MS mode using the same spot on the target.

Phosphorylation assay

The DLC1 GAP domain was produced as a GST fusion in *Escherichia coli* and prepared as described in [15]. The GAP domain (1 μg) was incubated for 30 min at 30 °C with purified PKD1 in the presence of 20 μM ATP in kinase buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 2 mM DTT). The reaction was stopped by addition of sample buffer, and proteins were separated by SDS-PAGE and analyzed by immunoblotting.

In vitro GAP assays

HEK293T cells were transiently transfected in triplicates with plasmids encoding for GFP-DLC1 WT, S807A, S807D, S807E and K714E. Proteins were immunoprecipitated from WCE with GFP-specific mouse mAb and Protein G agarose. *In vitro* GAP assays were done as described previously [16]. In brief, recombinant RhoA (10 μg) was loaded with γ^{32}-P-GTP (10 μCi) in 30 μl loading buffer for 5 min at 37 °C. Loading was stopped by addition of 1.5 μl 1 M $MgCl_2$. 10 μl RhoA loaded with γ^{32}-P-GTP were added to 40 μl reaction buffer containing the immobilized DLC1 variants. GST-RhoA-bound γ32-P-GTP was measured at 0 min (100% GTP) and after 10 min at 20 °C (GTP remaining %). The reaction was stopped by adding 10 μl of the reaction mix to 1 ml cold washing buffer, and aspiration with a vacuum manifold. Filters were washed with 7 ml washing buffer and radioactivity was detected by scintillation counting.

Random migration assays

MCF7 cells were transfected using the Amaxa nucleofector (Kit V; program P-020). The next day, 10^5 cells were seeded into the upper well of a Transwell (8.0 μm; Costar) and allowed to migrate for 24 h. Both the upper and the lower chamber contained medium supplemented with 10% FCS. Cells on the upper side of the membranes were removed using a cotton swab and cells on the underside were fixed in 4% PFA and stained with 0.1% crystal violet. Cells were counted in five independent microscopic fields at a 20-fold magnification.

Colony formation assays

MCF7 cells were transfected with plasmids encoding for GFP-DLC1 variants or empty vector. 1 day later, cells were replated in triplicate dishes (1×10^5/6-well dish) and selected with 0.8 mg/ml G418 for 2 weeks. After staining of the cells with crystal violet, pictures were taken with a binocular loupe. Colonies with a diameter larger than 200 pixel² were counted with WCIF ImageJ.

Results and discussion

To identify novel phosphorylation sites within DLC1, we performed MALDI-TOF mass spectrometry analysis of Flag-tagged DLC1 ectopically expressed in HEK293T cells and purified with M2 agarose. To enrich for phosphorylated peptides, samples were subjected to immobilized metal affinity chromatography prior to analysis. A peptide with the parent mass $[M+H]^{+1}$ of 925.3 corresponding to amino acids 804–810 (RENpSSPR) in DLC1 was identified, which showed the characteristic loss of phosphoric acid (~98 Da) (Fig. 1). Tandem MS unambiguously identified serine 807 to be phosphorylated. This phosphosite was of particular interest as it is contained within the GAP domain of DLC1, raising the possibility that phosphorylation at serine 807 may be directly involved in the regulation of GAP activity. We furthermore noted that this site matches the consensus motif of PKD (L/I/VxRxxS/T; Fig. 2A), a kinase that we have previously shown to phosphorylate DLC1 on serines 327 and 431 [10]. To investigate whether PKD also phosphorylates DLC1 on this site, we raised a phospho-S807-specific antiserum by immunizing rabbits with the corresponding phosphopeptide. Indeed, in immunoblots the serum readily detected the wild type (WT) DLC1 protein when coexpressed with PKD1 in HEK293T cells but failed to crossreact with a DLC1-S807A mutant, proving specificity of the antiserum (Fig. 2B). Expression of PKD2 and PKD3 equally enhanced DLC1 detection with the antiserum (Fig. 2C), thus identifying the PKD family of serine/threonine kinases as upstream kinases of DLC1 at serine 807. To prove that PKD phosphorylates serine 807 directly, we expressed the DLC1 GAP domain as a GST fusion in *E. coli* and subjected the purified protein to a phosphorylation assay with recombinant PKD1. Immunoblotting with the pS807-antibody demonstrated that PKD1 can phosphorylate DLC1 at this site *in vitro* (Fig. 2D).

To address whether endogenous PKD was involved in phosphorylating DLC1 at serine 807 we used phorbol-12,13-dibutyrate (PDBu), an analog of diacylgycerol, to stimulate cellular PKD activity. PDBu activates novel PKCs, which in turn phosphorylate PKD within the activation loop. HEK293 cells stably expressing GFP-tagged DLC1 in an inducible manner (FlpIn-DLC1 cells; [10]) were treated with doxycycline to switch on DLC1 expression and then stimulated with PDBu, followed by immunoblotting of whole cell lysates with the p807 antiserum. PDBu stimulation strongly increased DLC1 detection, and thus phosphorylation at serine 807 (Fig. 2E). This was inhibited by preincubation of cells with specific pharmacological inhibitors of the PKC/PKD pathway (Fig. 2E). Gö6983 inhibits novel PKCs whereas Gö6976 inhibits PKD directly. Staurosporine, which blocks a wide range of kinases including PKCs and PKD, was used as control. To verify the functionality of the inhibitors, lysates were immunoblotted with an antibody that recognizes autophosphorylated PKD1 and PKD2 (anti-(P)S910) and can thus be used to monitor the activation state of PKD [11]. Compared with unstimulated cells, PDBu treatment strongly increased PKD autophosphorylation and this was efficiently blocked with each of the inhibitors (Fig. 2E). Together these results indicate that PKD phosphorylates DLC1 on serine 807 in a novel PKC-dependent manner. PKD has been reported to be activated downstream of RhoA [17,18], raising the possibility that RhoA feeds back on its own negative regulator. We therefore coexpressed DLC1 together with constitutively active Rho-G14V. The presence of active Rho enhanced PKD activation as judged by immunoblotting of cell lysates with the PKD-(P)S910 antibody and concomitantly enhanced DLC1 phosphorylation at serine 807 (Fig. 2F). This phosphorylation could be blocked by incubation of cells with the PKC/PKD Gö6976 and Gö6983 inhibitors (Fig. 2F),

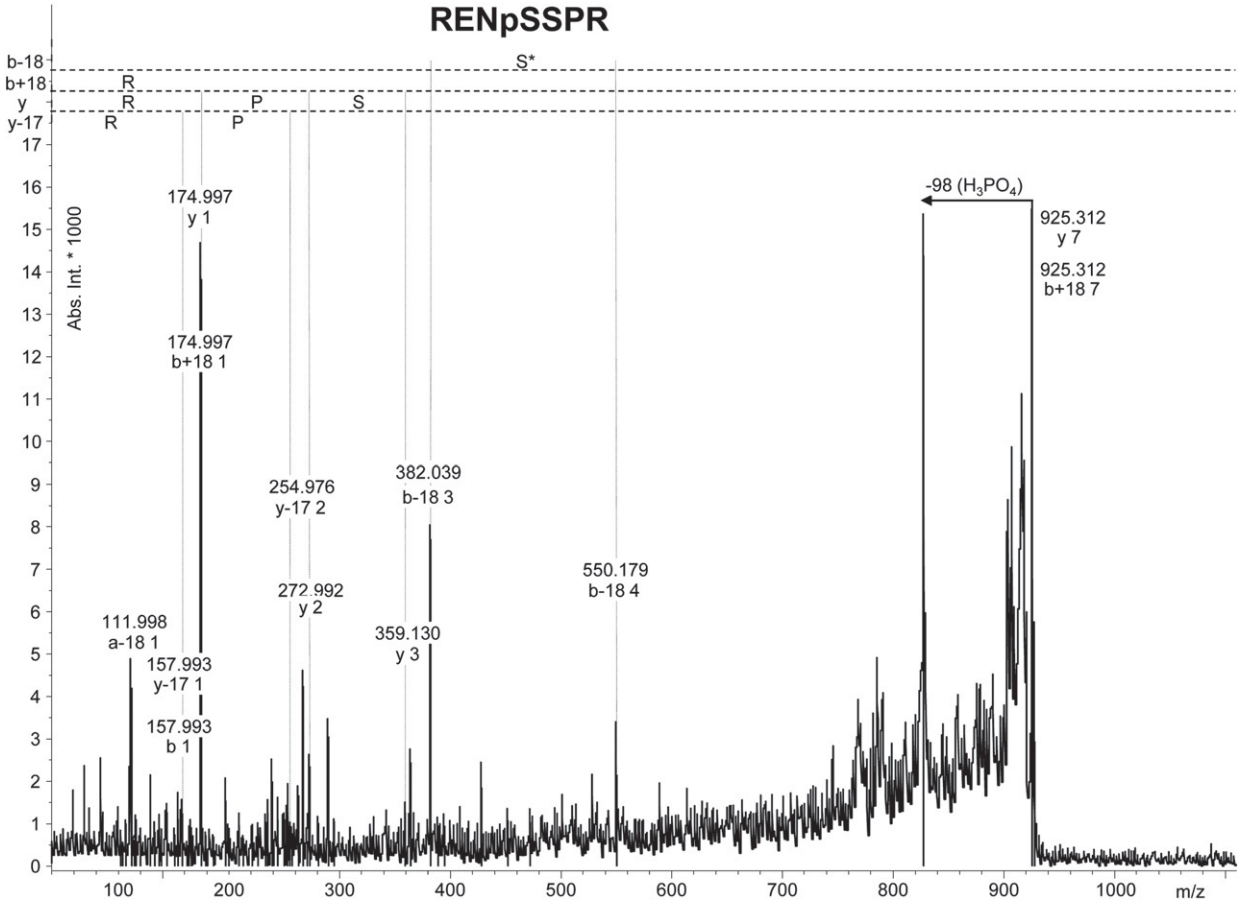

Fig. 1 – Identification of DLC1 phosphorylation on serine 807. Flag-tagged DLC1 was purified from HEK293T cell lysates transiently expressing the protein with M2 agarose and subjected to SDS-PAGE. The Coomassie-stained band corresponding to DLC1 was excised and digested with trypsin. Phosphopeptides were enriched by IMAC and then subjected to analysis by mass spectrometry. The spectrum shows the fragmentation pattern of the phosphopeptide RENpSSPR corresponding to amino acids 804–810. The loss of H_3PO_4 (98 Da) and the presence of b_3(-18)- and b_4(-18)-ions confirm phosphorylation on serine 807.

indicating a link between Rho activation and DLC1 phosphorylation states.

The fact that serine 807 lies within the DLC1 GAP domain prompted us to test whether *in vitro* GAP activity was altered by phosphorylation at this site. To this end, DLC1 wild type and the 807 phosphorylation site mutants were immunoprecipitated from HEK293T cell lysates and subjected to *in vitro* GAP assays with recombinant RhoA loaded with radiolabeled GTP. In this assay, GTP hydrolysis rates were similar in all cases (Fig. 3A). DLC1 is also known to possess *in vitro* GAP activity for Cdc42, although to a lesser extent [4,19]. However, measurements with Cdc42 as a substrate did not reveal any differences (data not shown), indicating that–at least *in vitro*–serine 807 phosphorylation does not appear to impact on DLC1's GAP activity. Using a RhoA biosensor, cellular GAP activities of the DLC1 807 serine-to-alanine exchange mutant and a triple mutant additionally containing mutations of the previously described PKD phosphorylation sites (S327/431/807A) were also similar to that of the wild type protein (Fig. S1).

We next tested whether phosphorylation at serine 807 affects the biological activity of DLC1. Ectopic expression of DLC1 is known to increase random migration of MCF7 cells in a GAP-dependent manner, most likely by generating an imbalance between Rho and Rac activities [16,20]. To determine whether serine 807 phosphorylation has an effect on this property of DLC1, we expressed wild type DLC1, and the S807A, S807D and S807E mutants in MCF7 cells and analyzed their motility in transwell assays in the absence of a chemotactic gradient. In accordance with previous results, DLC1-WT dramatically enhanced the number of migrated cells compared to the control (Fig. 3B). Compared to the wild type protein, the S807A mutant was more potent at stimulating migration (Fig. 3B), whereas no statistically significant differences were observed in the case of the S807D and S807E mutants (data not shown). To investigate the involvement of the Rho pathway in this assay, we co-expressed active RhoA (RhoA-Q63L). Compared to the control, RhoA-Q63L alone slightly reduced the number of migrated cells (Fig. 3C). Importantly, RhoA-Q63L potently inhibited DLC1-induced random migration, making it very likely that ectopically expressed DLC1 stimulates cell motility primarily through Rho inactivation (Fig. 3C).

DLC1 has further been shown to inhibit colony formation of several cancer cell lines. To address the impact of serine 807 phosphorylation in this context, MCF7 cells were transfected with the different DLC1 expression constructs and subjected to antibiotic selection. After 2 weeks, cells were fixed and stained, and the number of colonies was scored. Consistent with the

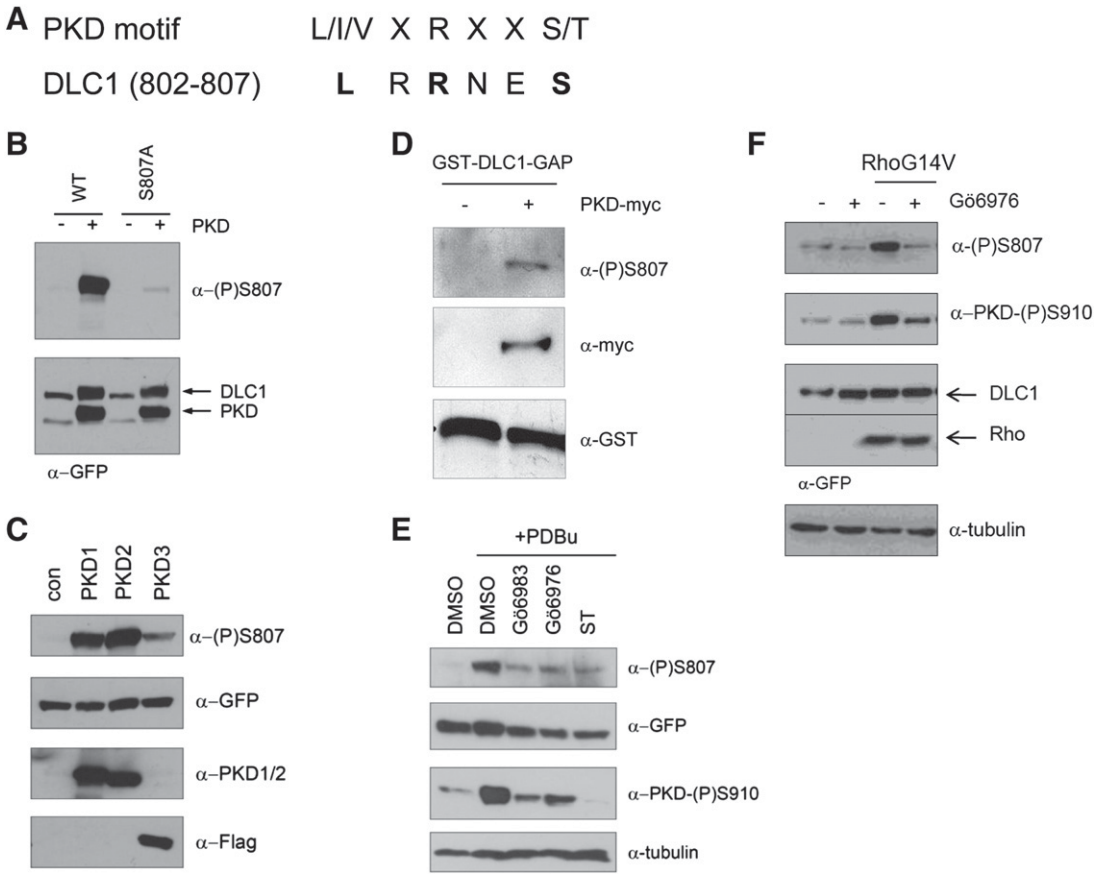

Fig. 2 – PKD phosphorylates DLC1 on serine 807. (A) PKD consensus motif and DLC1 sequence alignment (amino acids 802–807). (B) HEK293T cells were transiently cotransfected with expression plasmids encoding GFP-tagged DLC1-WT or DLC1-S807A and empty vector (−) or GFP-tagged PKD1 (+), respectively. Phosphorylation of DLC1 was detected by immunoblotting of whole cell extracts (WCE) with DLC1-(P)S807-specific antibody (upper panel). Expression of PKD1 and DLC1 variants was verified with GFP-specific antibody (bottom panel). (C) HEK293T cells were transiently cotransfected with expression vectors encoding GFP-DLC1 and PKD1, PKD2, Flag-PKD3 or empty vector as a control. Phosphorylation of DLC1 was detected by immunoblotting with DLC1-(P)S807-specific antibody (upper panel). Expression of DLC1 was verified with a GFP-antibody (panel 2), expression of PKD1/2 with the PKD-specific antibody C20, and PKD3 with Flag-specific antibody (bottom panels). (D) The GST-tagged DLC1 GAP domain purified from *E. coli* was incubated with recombinant myc-tagged PKD1 in the presence of ATP. Proteins were separated by SDS-PAGE and *in vitro* phosphorylation of the DLC1 GAP domain was analyzed by immunoblotting with DLC1-(P)S807-specific antibody (top panel). Loading was controlled with myc- and GST-specific antibodies (bottom panels). (E) Flp-In-DLC1 cells were treated with 10 ng/ml doxycycline over night and then incubated with 1 μM staurosporine (ST), 5 μM Gö6983, 5 μM Gö6976 or solvent (DMSO) for 90 min and then left untreated or treated with 1 μM PDBu for 15 min. WCE were subjected to SDS-PAGE and phosphorylation of DLC1 was detected by immunoblotting with DLC1-(P)S807-specific antibody (upper panel). Expression of DLC1 was verified by reprobing with a GFP-specific antibody (panel 2). PKD autophosphorylation was detected with PKD-(P)S910 antibody (panel 3) and equal loading was confirmed with a tubulin-specific antibody (bottom panel). (F) HEK293T cells were transiently cotransfected with expression vectors encoding GFP-DLC1 and GFP-RhoA-G14V or empty vector as a control. The next day cells were treated with 5 μM Gö6976 for 2 h (+) or DMSO (−). WCE were subjected to SDS-PAGE and DLC1 phosphorylation was detected by immunoblotting with DLC1-(P)S807-specific antibody (upper panel). PKD autophosphorylation was detected with PKD-(P)S910 antibody (panel 2). Expression of DLC1 and RhoA-G14V was verified with a GFP-specific antibody (panel 3) and equal loading was confirmed with a tubulin-specific antibody (bottom panel).

random motility assays, the DLC1-S807A mutant was significantly more active than the wild type protein at inhibiting colony formation independently of colony size (Fig. 4A, B; Fig. S2), while DLC1-S807D and DLC1-S807E yielded a similar number of colonies as the wild type protein (Fig. S2). Of note, the DLC1-K714E mutant also caused a slight reduction in the number of colonies, which has been attributed to GAP-independent functions of the protein (Fig. S2). Taken together, our data suggest that PKD-mediated DLC1 phosphorylation at serine 807 negatively regulates DLC1 cellular function.

Although we did not find any changes in GAP activity towards RhoA *in vitro*, the DLC1 S807A phosphorylation site mutant possessed higher activity in cellular assays. As our *in vitro* experiments were done with recombinant RhoA and Cdc42, it

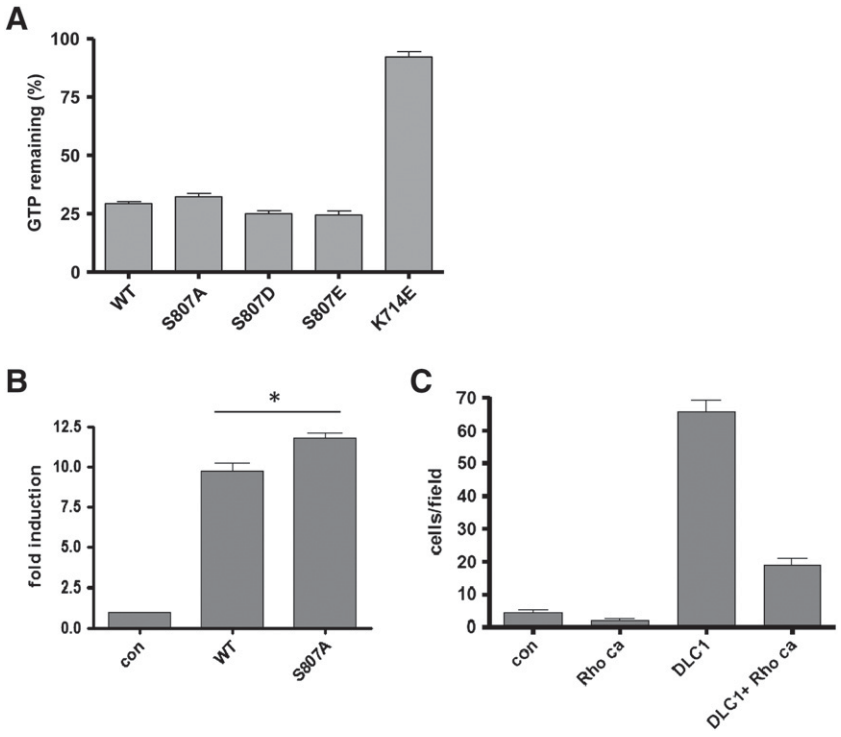

Fig. 3 – Serine 807 phosphorylation modulates DLC1 cellular mol activity. (A) GFP-tagged DLC1-WT, DLC1-S807A, DLC1-S807D, DLC1-S807E and DLC1-K714E were immunoprecipitated from lysates of HEK293T cells transiently expressing the proteins and incubated with GST-RhoA loaded with radiolabeled GTP. GST-RhoA-bound γ32-P-GTP was measured at 0 min (100% GTP) and after 10 min at 20 °C (GTP remaining %). The graph shows the average of three independent experiments each performed with triplicate samples; error bars represent SEM. (B) MCF7 cells were nucleofected with expression plasmids encoding GFP-tagged DLC1-WT, DLC1-S807A or empty vector as a control (con). The next day, cells were seeded into transwells containing medium supplemented with 10% FCS in both the upper and the lower chamber. Equal expression of the DLC1 variants was verified by measurement of GFP fluorescence of the remaining cells by FACS analysis (not shown). After 24 h, migrated cells in five independent microscopic fields per filter were counted (20-fold magnification). Data show the mean of four independent experiments performed with duplicate filters, the empty vector control was set as 1. Error bars represent SEM; $^{*}p = 0.015$. (C) MCF7 cells were nucleofected with expression plasmids encoding GFP-tagged RhoA-Q63L (ca), DLC1, and DLC1 in combination with RhoA-Q63L or empty vector as a control (con). The random migration assay was performed as described in B. Data show a representative experiment.

cannot be ruled out that phosphorylation specifically modulates substrate specificity towards another GTPase. For example, the specificity of MgcRacGAP switches from Rac and Cdc42 to Rho in response to phosphorylation by Aurora B kinase [21]. However, DLC1 S807 phosphorylation site mutants did not demonstrate any activity towards a genetically encoded Rac1 biosensor in transient transfection experiments (data not shown). Alternatively, phosphorylation can impact on protein localization and/or protein-protein interactions. DLC1 recruitment to focal adhesions is thought to be a prerequisite for biological activity [22,23], but the subcellular localization of the DLC1 S807 phosphorylation site mutants was undistinguishable from that of the wild type protein (data not shown). Recently, the DLC1 GAP domain was reported to be inhibited by interaction with the p120RasGAP protein [24]. In pulldown experiments, we did not observe any differences in p120RasGAP association with the DLC1 S807 phosphorylation site mutants (data not shown), but it remains possible that complex formation with other still to be identified protein partners is affected by DLC1 phosphorylation on this site. In contrast to PKD-mediated phosphorylation of serines 327 and 431, phosphorylation of serine 807 did not influence 14-3-3 protein binding (Fig. S3). Elucidation of the precise biochemical mechanism by which DLC1 phosphorylation on serine 807 modulates its activity may thus require screening for interaction partners at the proteomic level.

PKD is a family of serine/threonine kinases for which only a few physiological substrates have been identified thus far. The function of PKD has been studied best at the Golgi complex where it is known to regulate vesicular traffic to the plasma membrane [25]. More recent studies by us and other labs suggest that PKD has additional functions associated with the regulation of cell shape, movement and invasion [26–29]. The identification of the RhoGAP and tumor suppressor protein DLC1 as a PKD substrate thus creates a novel link between PKD, Rho signaling and cell transformation. The fact that PKD is activated downstream of Rho under certain conditions, which in turn would result in DLC1 inhibition by PKD-mediated phosphorylation implicates a feedback mechanism allowing amplification of Rho signaling. We previously reported that PKD inhibits DLC1 cellular function through phosphorylation of serines 327 and 431 [10]. Here we provide evidence that PKD additionally phosphorylates serine 807 contained within the DLC1 GAP domain. The fact that PKD negatively controls DLC1 through

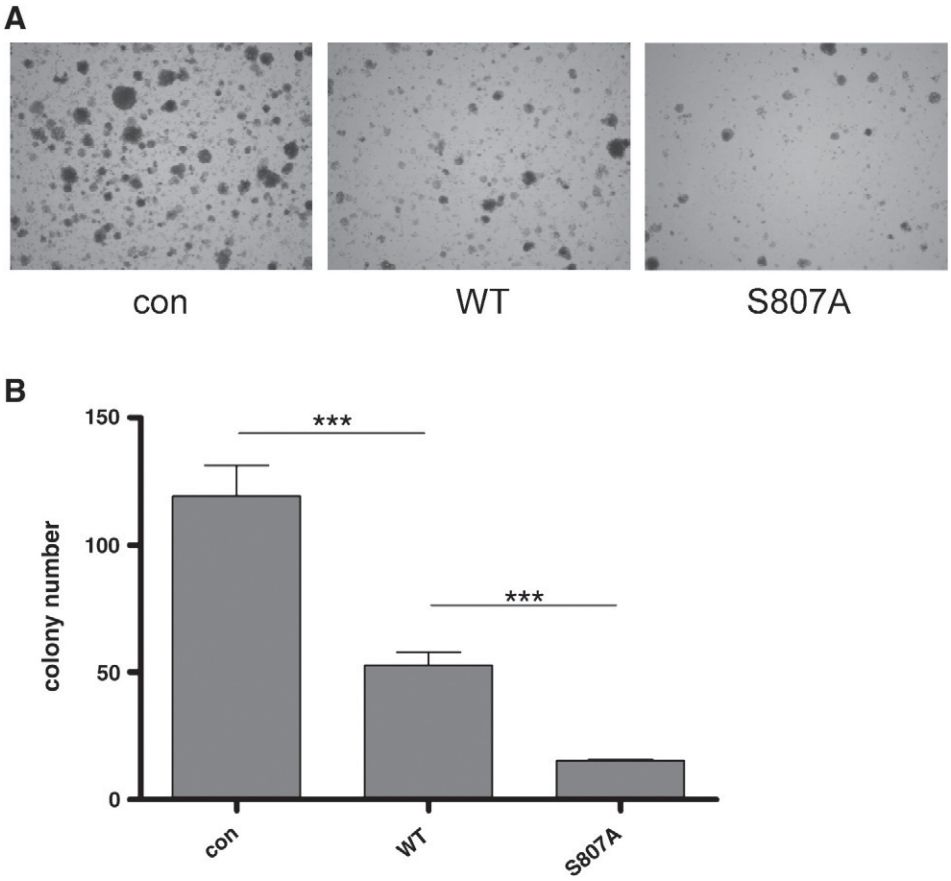

Fig. 4 – Expression of a DLC1-S807A phosphorylation site mutant inhibits MCF7 colony formation. (A) MCF7 cells were transfected with plasmids encoding GFP-tagged DLC1-WT, DLC1-S807A or empty vector (con). Cells were replated (1×10^5 per 6-well dish) and selected with 0.8 mg/ml G418 for 2 weeks. After staining of the cells with crystal violet, pictures were taken with a binocular loupe. (B) Quantification of colonies shown in A. Colonies with a diameter larger than 200 pixel² were counted with WCIF ImageJ. The data shown correspond to a representative experiment with triplicate wells ($n=2$). Error bars represent SEM; $^{***}p=0.0002$.

phosphorylation on multiple sites underscores the necessity for tight control of DLC1 cellular function to ensure correct Rho signaling.

Supplementary materials related to this article can be found online at doi: 10.1016/j.yexcr.2010.11.003.

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