Biochemical and structural characterization of Rho family proteins and their interaction with Plexin-B1 and IQGAP1

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Düsseldorf, May 2013

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Introduction

1. Rho family proteins

Rho proteins (ras homologous) family is one of the major families of the Ras superfamily, which is the largest group of guanine nucleotide-binding proteins (GNBPs). The majority of Rho family members can also be termed as small GTPases, since they have a low molecular weight of 20-30 kDa and are able to bind and with some exceptions to hydrolyze GTP. Like other small GTPase, Rho GTPases act as molecular switches by cycling between a GTP-bound (active) and a GDP-bound (inactive) state. They are mainly involved in the reorganization of the cytoskeleton, the control of cell growth and the regulation of gene expression (Mackay and Hall, 1998; Sander and Collard, 1999). The family of Rho GTPases comprises 23 members in mammals, which exhibit a sequence homology of 40 to 95%. On basis of their primary sequence homology and biological function, Rho proteins can be divided into six subgroups (Wennerberg and Der, 2004) (Fig. 1).

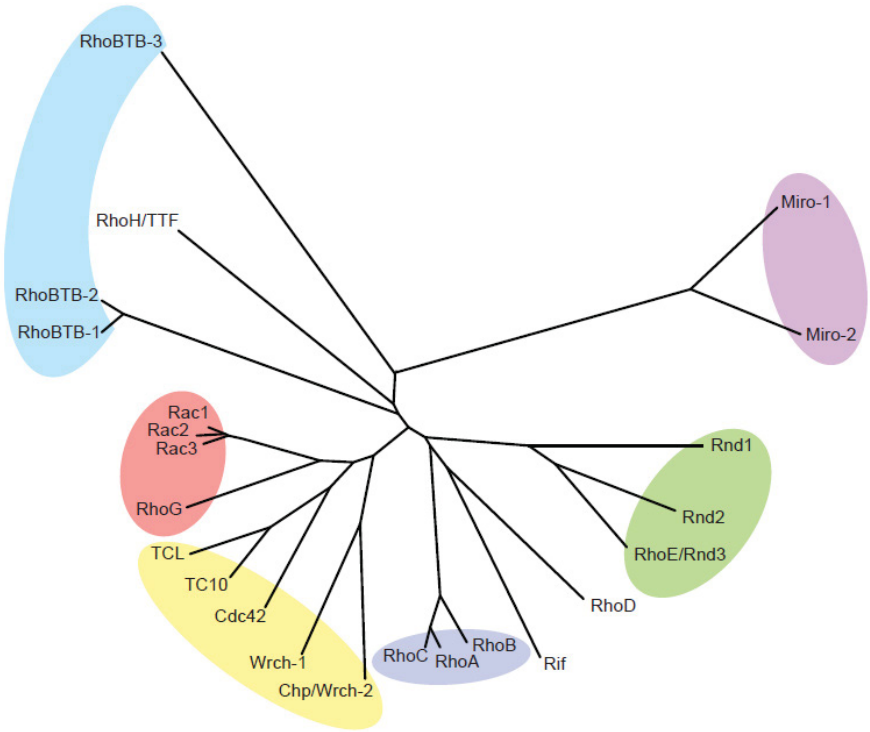

Figure 1: Phylogenetic tree of the Rho family protein. The 23 members of the Rho family can be divided into six main subgroups: Rho-related, Rac-related, Cdc42-related, Rnd-related, RhoBTB-related and Miro-related, where the proteins RhoD, Rif and RhoH cannot assign as defined subgroup. Modified from (Wennerberg and Der, 2004).

1.1. Role in cellular processes and human diseases

Rho family proteins are involved in the regulation of a broad spectrum of cellular processes. The best characterized members of the Rho family are RhoA (ras homologous A), Rac1 (ras related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). All three are involved in signaling pathways such as cell migration, adhesion, and gene expression (Hall, 2012). They regulate the organization of the actin cytoskeleton, observed in the cell morphological changes. Activation of RhoA leads to the formation of long actin fibers (stress fibers) and focal adhesion. Rac1 induces lamellipodia formation and Cdc42 activation form finger-like filopodia (Hall, 1998; Schmitz et al., 2000). In contrast to the oncogenic Ras proteins, which are constitutively activated by point mutation and thus contribute to tumorigenesis, to date no mutations in Rho proteins have been discovered in tumors, with the exception of RhoH/TTF, which has been shown to be involved lymphoma development (Dallery et al., 1995; Preudhomme et al., 2000) by nonrandom rearrangements of the *RhoH* gene. Nevertheless, this family of proteins plays a central role tumor progression and metastasis (Jaffe and Hall, 2002; Price and Collard, 2001; Schmitz et al., 2000). This property is due to an increased level of expression and the altered activity of regulatory proteins.

Several signaling pathways, in which Rho proteins are involved, are well studied, including morphogenesis, phagocytosis, pinocytosis, cytokinesis, axonal growth, cell-cell adhesion, cell-substrate adhesion, cell polarity, cell cycle and cell locomotion (Etienne-Manneville and Hall, 2002; Hall, 2012). The latter is critical for embryonic development, as well as immune response, wound healing, tumor formation and metastasis (Ridley, 2004; Titus et al., 2005; Vega and Ridley, 2008). The different subcellular localization of Rho proteins is associated with their biological functions.

The regulation of Rho proteins could be critical for physiological integration of cell. Thus, dysregulation or dysfunction of Rho signaling pathways results in severe human diseases, such as cancer, mental retardation and immunological disorders (Ambruso et al., 2000; Nadif Kasri and Van Aelst, 2008; Sahai and Marshall, 2002). In addition, Rho proteins and their regulators and effectors are related to a number of clinical symptoms such as neurodegenerative diseases and particularly many X-linked genetic diseases (Boettner and Van Aelst, 2002; Ramakers, 2002).

1.2. Structural features and biochemical properties

The central motif of Rho proteins is the G-domain, which is responsible for characteristic functions, nucleotide binding and hydrolysis. Different from other GTPases, Rho proteins contain helix α3, also called as insert helix. The core domain is highly conserved among Rho family members and consists of a central six stranded β-sheets and five α-helices, which are linked by 10 loop regions (Fig. 2). Five of these loops (G1 - G5) are for the specificity and high affinity binding of the nucleotide responsible (Bourne et al., 1991; John et al., 1990; Schmidt et al., 1996; Via et al., 2000). The G1-motif, called also as P-loop, has the consensus sequence GxxxxGK(S/T) and is main contributor to the high affinity binding of the nucleotide and also involved in magnesium ion (Mg^{2+}) coordination (Saraste et al., 1990). The G2 and G3 motifs, also called as switch I and switch II regions, undergo conformational changes in different nucleotide bound states (Fig. 2). The switch regions are the recognition sites for the interaction of regulators and effector molecules (Dvorsky and Ahmadian, 2004). The G4 and G5 motifs are responsible for the recognition of the guanine (Schmidt et al., 1996; Zhong et al., 1995).

Except for Chp, RhoBTB1, RhoBTB2 and Miro1 and Miro2, Rho proteins exhibit at their C-terminus a hyper variable region (HRV) containing a CAAX motif (C is cysteine, A is any aliphatic amino acid, and X is any amino acid). This region is responsible for membrane anchoring by posttranslational modification such as farnesylation, geranyl-geranylation or palmitoylation.

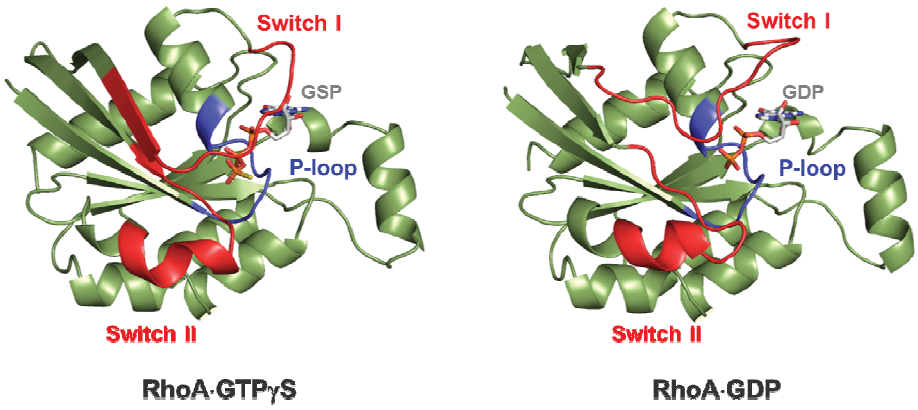

Figure 2: Conformational changes of RhoA according the nucleotide-bound state. The figure shows that Rho in the active GTP-bound state (here GTPγS as non-hydrolysable analogue) (Ihara et al., 1998) differ from the inactive GDP-bound form (Wei et al., 1997). The conformational changes, however, are limited to the switch regions (switch I and switch II, shown here in red). The phosphate-loop is almost identical in both states (P-loop, here shown in blue).

The biochemical properties of Rho proteins include nucleotide binding, exchange of guanine nucleotides (GDP by GTP) and the hydrolysis of GTP. Based on these properties, they can be divided into two major classes (Jaiswal et al., 2013b). A conventional class including RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, RhoG, Cdc42, TC10 and TCL, which exhibit extremely slow nucleotide exchange and a relatively fast GTP hydrolysis keeping them in their GDP-bound (inactive) state under resting conditions. While an non-conventional class includes those Rho proteins, which persist in GTP-bound state due to their faster nucleotide exchange and a slower GTP hydrolysis rates such as RhoD and Rif, or their lack of a GTPase activity such as Rnd1, Rnd2, Rnd3 and TTF/RhoH. The residual Rho proteins can also be arranged under the non-conventional class. Wrch1 and Chp are characterized by the presence of N-terminal proline-rich region. RhoBTB proteins contain additional to G-domain two BTB domains and NLS, nuclear localization sequence. Miro proteins are mitochondrial RhoGTPases containing two G domains, in which only the N-terminal G domain have certain similarity to typical Rho proteins. Additionally, Miro proteins neither have the Rho insert helix nor the C-terminal CAAX motif.

1.3. Regulation of the GTPase cycle of Rho proteins

Most of Rho proteins as indicated above function as molecular switches that cycle between an active GTP-bound and inactive GDP-bound state (Fig. 3). This function is achieved by their very slow intrinsic GDP/GTP exchange and GTP-hydrolysis reactions. However, a strict control of these reactions is critical for the signal transduction processes, and requires regulatory proteins including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitor (GDI). GEFs stimulate the dissociation of the bound nucleotide by several orders of magnitude, resulting in accumulating of Rho proteins in the GTP-bound active state (Jaiswal et al., 2013a; Pan and Wessling-Resnick, 1998; Rossman et al., 2005; Whitehead et al., 1997). GAPs stimulate the intrinsic hydrolysis by several orders of magnitude, thus leading to a rapid inactivation of Rho proteins (Bernards, 2003; Boguski and McCormick, 1993; Ligeti et al., 2012; Scheffzek and Ahmadian, 2005; Scheffzek et al., 1998a). GDI inactivates Rho proteins by inhibiting the dissociation of the nucleotide or by translocating them from their place of signal transduction (DerMardirossian and Bokoch, 2005; Olofsson, 1999). Rho proteins in their active GTP-bound conformations bind to

downstream effectors, resulting in transmitting the signal (Bishop and Hall, 2000; Herrmann, 2003).

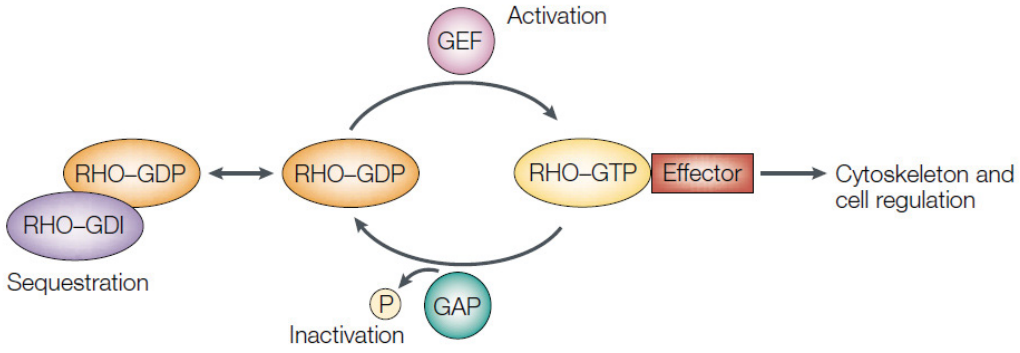

Figure 3: Rho Proteins as molecular switches. Rho proteins bind either GTP or GDP. Inefficient intrinsic functions of Rho proteins require tight regulation by proteins that speed up these processes. Guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP. GTPase activating proteins (GAPs) promote hydrolysis of GTP to GDP and inorganic phosphate (P). When bound to GDP, they can be sequestered in the cytoplasm by guanine nucleotide dissociation inhibitors (GDIs). When bound to GTP, they interact with effectors. Modified from (Sahai and Marshall, 2002).

1.4. Interacting partners of Rho proteins

The signal transduction of Rho proteins is achieved through their interaction with effector proteins (Bishop and Hall, 2000; Schmitz et al., 2000; Van Aelst and D'Souza-Schorey, 1997). An important feature of the effectors is their ability to bind to the GTP bound state of Rho proteins resulting in effector activation. Due to the variety of important cellular processes in which the Rho proteins are involved, the identification and characterization of the target effector has been intensively investigated in the case of RhoA, Rac1 and Cdc42 (Bishop and Hall, 2000; Van Aelst and D'Souza-Schorey, 1997).

According to their Rho binding domains (RBDs) the Rho effectors can be divided into three classes (Bishop and Hall, 2000; Fujisawa et al., 1998). Class I includes Rho effector homology proteins (REM) such as Rhotekin, Rhophilin and PRK1/PKNα, which possess a conserved HR1-like domain. ROK-kinectin homology proteins (RKH) comprise class II of Rho effectors, which includes ROCK and Kinectin, whereas citron kinase is known as class III. In contrast to Rho effectors, many effectors of Rac and Cdc42 contain a conserved GTPase binding consensus sequence, called CRIB (Cdc42/Rac interactive binding) motif (Burbelo et al., 1995). The p21 activated kinase (PAK), the Wiskott-

Aldrich syndrome protein (WASP) and the activated Cdc42-associated tyrosine kinase (ACK) are the most investigated Rac and Cdc42 effectors, which contains CRIB-motif.

The resolution of several of structures of Rho proteins in complex with regulators and downstream effectors added valuable insights into the structure-function relationships between Rho proteins and their interacting partners. The comparison of these structures has revealed common characteristics of the interactions (Dvorsky and Ahmadian, 2004). Accordingly, the switch regions are most likely the recognition sites for various Rho interacting proteins, which contact other regions that are specific to their function (Fig. 4).

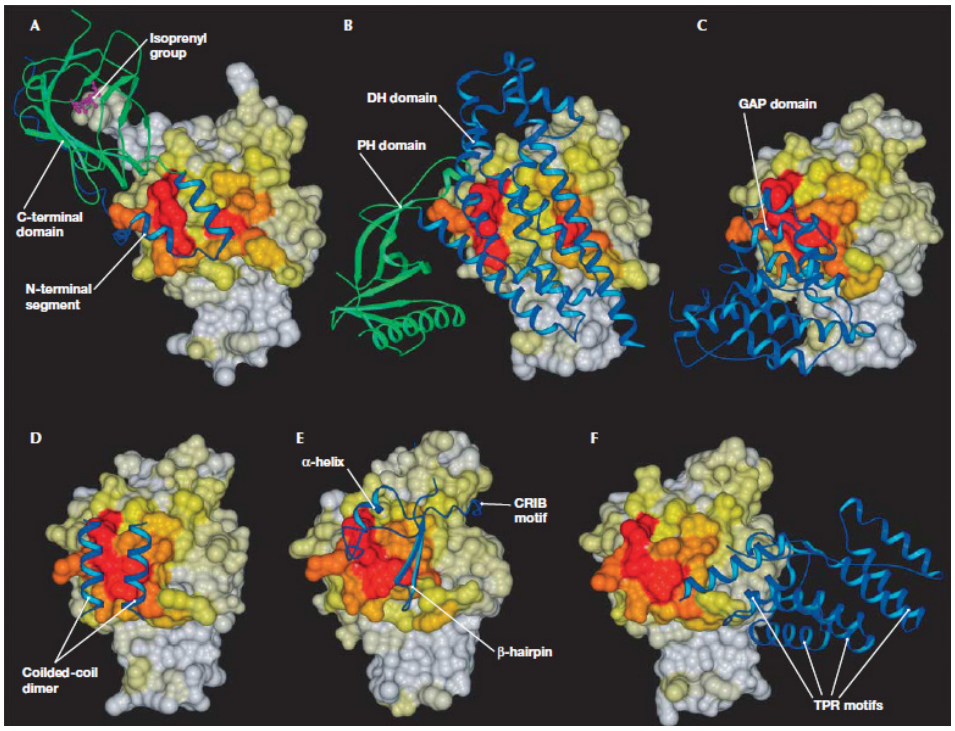

Figure 4: Comparison of complex structures of Rho proteins with different binding partners. (A) Cdc42·GDP·GDI. (B) RhoA·Dbs. (C) RhoA·GDP·AlF_4^-·GAP. (D) RhoA·GppNHp·ROCK. (E) Cdc42·$GppCH_2p$·WASP. (F) $Rac1^{Q61L}$·GTP·$p67^{phox}$. Modified from (Dvorsky and Ahmadian, 2004).

All regulatory proteins also contact the α3-helix (aa 99-104; RhoA numbering). GDIs bind additionally the prenyl moiety at the C-terminus. By contrast, effector proteins exhibit various interaction patterns depending on the effector type and predominantly bind to the amino (N)-terminal part of Rho proteins or the region around the α5-helix (aa 167-179 of RhoA). The mammalian diaphanous (mDia)-related Formin also contacts the insert helix of the RhoC (Rose et al., 2005). A different activation mechanism has been implicated for the Rho-specific effectors PKN and ROCK, which use other domains to bind cooperatively to the sites outside of the switch regions of RhoA (Blumenstein and Ahmadian, 2004). Accordingly, in addition to the switch regions other regions are needed for specifying the interaction of Rho proteins with a distinct binding partner.

Additional to the classical regulators and effectors, many modulators and scaffold proteins have been shown to be important interacting partners of Rho family proteins. Such molecules are Plexin-B1 and IQGAP1, their interactions with Rho proteins has been intensively investigated in the last ten years. Nevertheless, various aspects of these interactions are still unclear.

2. Plexins

Plexins belong to the best known and most widely expressed receptors of semaphorins. They are large, highly conserved transmembrane proteins that bind semaphorins and transduce their signaling in both vertebrates and invertebrates (Kruger et al., 2005; Tamagnone and Comoglio, 2000). Semaphorins were first characterized as axon guidance factors (Luo et al., 1993). The semaphorin family comprises more than 20 genes in vertebrates and another eight genes in invertebrates (Neufeld and Kessler, 2008). On the basis of their structural homology, semaphorines are divided into eight classes. They are referred to by SEMA followed by the number of subfamily and a letter defining the distinct member of the subfamily, i.e. SEMA4D. All semaphorins contain an N-terminal (~500 amino acids) Sema domain, which is important for their signaling. Activation of Plexins is triggered by specific semaphrine ligand and mediates repulsion by the axonal guidance (Tessier-Lavigne and Goodman, 1996). So far, nine Plexins has been discovered in vertebrates. They are divided into four subfamilies comprising four type A Plexins, three type B Plexins, Plexin-C1 and Plexin-D1 (Tamagnone et al., 1999) (Fig.5).

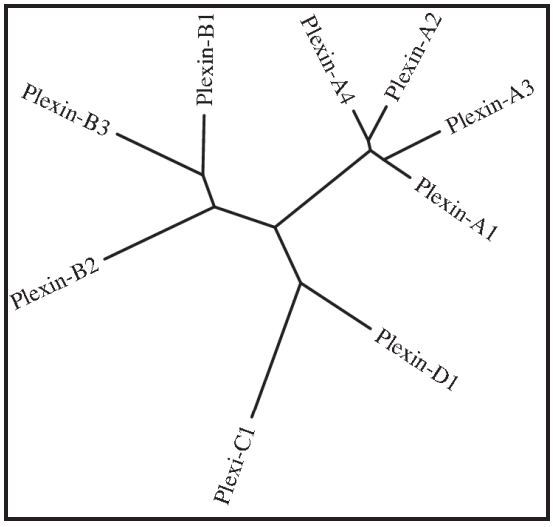

Figure 5: Phylogenetic tree of Plexins. The nine vertebrate Plexins can be divided into four main subgroups: Type A-related, type B-related, type C and type D. The tree was generated using full length sequences of the human Plexins with the application of phylogeny analysis tool (http://www.phylogeny.fr).

2.1. Role in development and human disease

Plexins function in diverse biological activities including the development and recovery of the neuronal and cardiovascular systems, heart disease and also skeletal and immune systems, and cancer related processes. In the development of mature nervous system Plexin signaling leads to correct targeting of neuronal cells (Ayala et al., 2007). They are also known to play an important role in the formation of dendrites (Shen and Cowan, 2010). The four subfamilies of Plexins are involved in different processes, i.e. Plexin-A4 mediates axonal repulsion (Suto et al., 2005), Plexin-B1 functions in neurite outgrowth and axonal growth cone collapse (Vodrazka et al., 2009), Plexin-D1 is involved in neuron connectivity (Pecho-Vrieseling et al., 2009), whereas the role of Plexin-C1 is unclear. Plexins were recently found to be involved in the development of cardiovascular system (Gitler et al., 2004), for example Plexin-A2 has been linked a spectrum of cardiovascular defects seen in the human syndromes (Goldmuntz and Emanuel, 1997) and also heart and vasculature defects (Brown et al., 2001). Plexin-D1 signaling has been shown to control vascular patterning (Gu et al., 2005).

Through their semaphorin ligands, Plexins were shown to be involved in cancer, either by mediating of tumor invasion and metastasis or as putative onco-suppressor genes (Basile et al., 2006; Bielenberg et al., 2004; Catalano et al., 2006; Christensen et al., 2005; Tomizawa et al., 2001). Plexins association with and activation of tyrosine kinase receptors has provided additional link to human tumors (Conrotto et al., 2004; Giordano et al., 2002; Swiercz et al., 2008). A molecular profiling of the human Plexin gene family in cancer provided evidence of the involvement of different Plexins in melanoma and pancreatic cancers (Balakrishnan et al., 2009). All four branches of Plexin family are involved in human cancer. For example Plexin-A4 promotes tumor progression and angiogenesis (Giordano et al., 2002). Mutations leading to loss of binding of Rho GTPases in Plexin-B1 were found in prostate and breast cancer (Wong et al., 2007). Plexin-C1 has been characterized as a tumor suppressor gene in skin cancer (Lazova et al., 2009). Plexin-D1 signaling was reported to downregulate tumor angiogenesis (Toyofuku et al., 2007).

Moreover Plexins are involved in several human diseases, as example Plexin-A1 and -A2 have been linked to Alzheimer (Good et al., 2004). Mutations in Plexin-A2 are associated with the development of schizophrenia (Mah et al., 2006). Plexin-B3 has been

related to other mental problems such as impaired verbal performance (Rujescu et al., 2007).

2.2. Domain organization and structural features

The Plexins are a homogeneous family of proteins, which are characterized by the presence of Sema domain (~500 amino acids) on their extracellular part, which is known to be responsible for the interaction with semaphorins. The Sema domain is followed by three small Plexin-semaphorin-integrin (PSI) domains, which contain only 50 amino acids, followed by three Ig-like, Plexins and transcription factors (IPT) domains. Additionally, B type Plexins contain in their extracellular domain a convertase cleavage site that is not found in other Plexins. The intracellular region of Plexins is highly conserved between different Plexins containing two remarkable features: (i) a split Ras-like GTPase-activating protein (GAP) domain sharing homology with the GAP domain of p120 RasGAP; (ii) a middle segment (~200 amino acids), which splits up the GAP domain and is responsible for binding of Rho family proteins (RBD) (Tong et al., 2007). Additionally, B type Plexins contain at their very C-terminus a PDZ (postsynaptic density-95, discs large, and zona occludens) binding motif (Fig. 6).

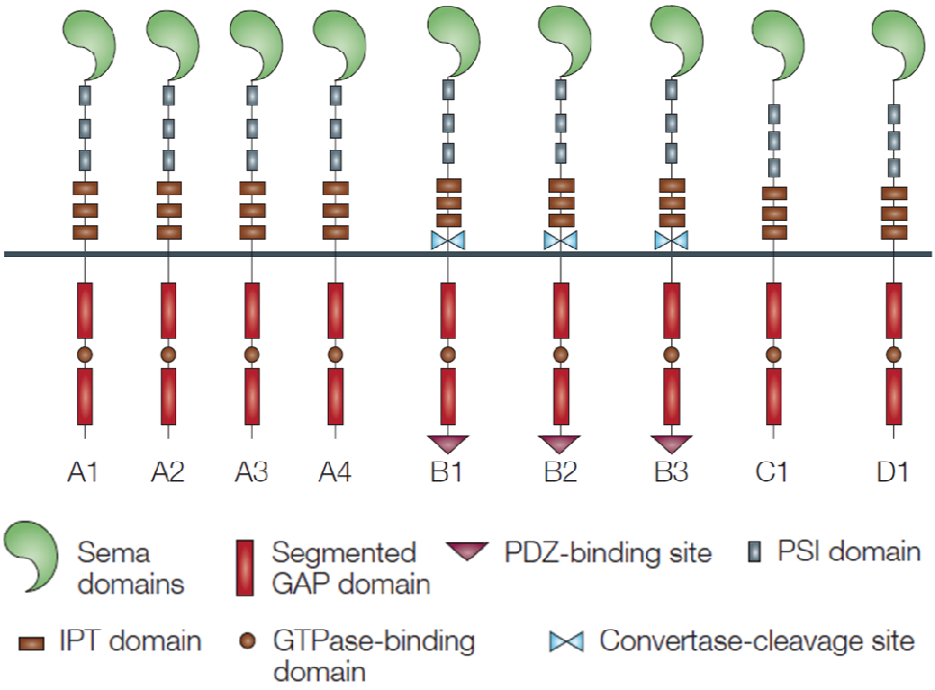

Figure 6: Domain organization of Plexins. Plexins are transmembrane receptors characterized by the presence of a split cytoplasmic Ras-like GAP domain and the presence of a sema domain at the extracellular region. Modified from (Kruger et al., 2005).

2.3. Signaling networks of Plexins with Rho family members

Through their Rho binding domain and PDZ motif, Plexins have tight relationship to Rho family proteins, which is involved in the repulsion of axonal growth cones (Fig. 7).

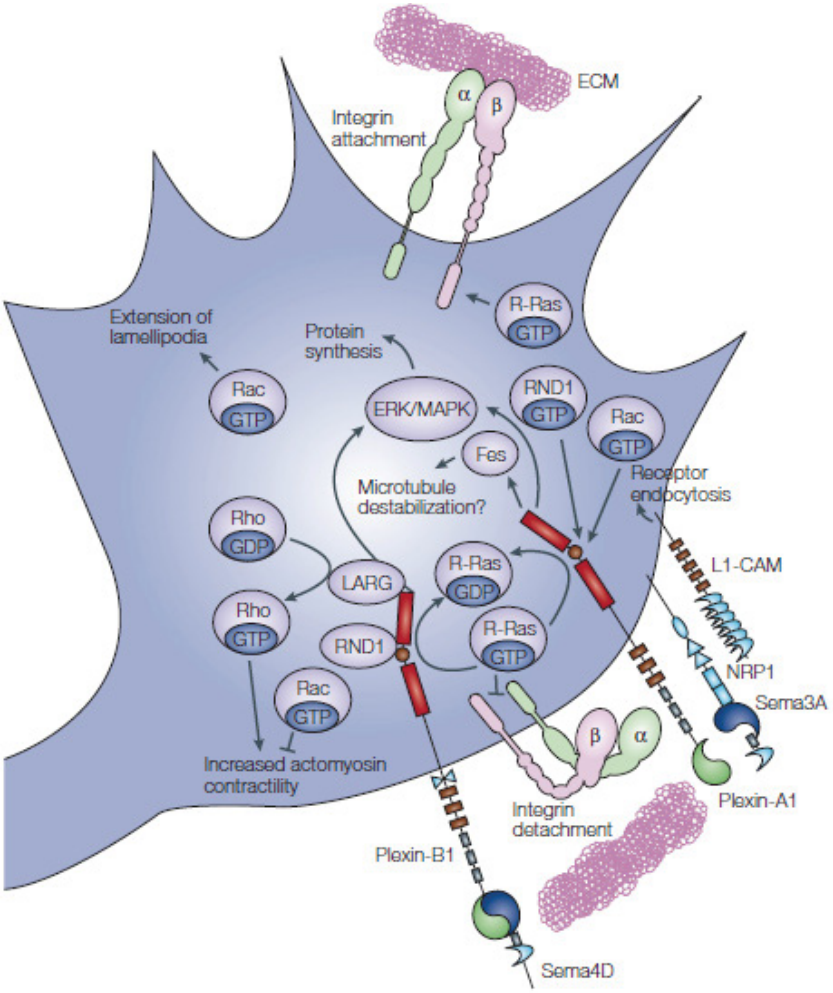

Figure 7: Plexins / Rho proteins signaling networks. Rho proteins are important components of Plexin downstream signaling at the edge of a growth cone. Plexin-B1 mediated activation of Rho by leukaemia-associated Rho-GEF (LARG) and PDZ-RhoGEF (PRG) results in contraction of actomyosin. Rnd1 is involved in the activation of the split GAP (GTPase-activating protein) domain of Plexin and promotes the GTP hydrolysis of R-Ras, leading to increase the GDP-bound inactive R-Ras level and decrease of the integrin-mediated attachment. In the absence of semaphorine, Rac1 promotes the activation of p21-activated kinase (PAK), whereas Sema4D binding sequesters active Rac1, suppressing PAK activity. These processes lead to the inhibition of axonal growth and promote turning. Modified from (Kruger et al., 2005).

Instead of directly binding to RhoA, Plexin-B1 has been shown to interact with leukaemia-associated Rho-GEF (LARG) and PDZ-RhoGEF (PRG) through its PDZ motif (Aurandt et al., 2002; Perrot et al., 2002; Swiercz et al., 2002). Binding of Semaphorin to Plexin-B1 stimulates the GEF activities of LARG and PRG, resulting in activation of

RhoA, which leads to repulsive growth of axons. In contrast, RhoA does not play any role in cell collapse mediated by Plexin-A1 (Turner et al., 2004).

The activation of Rac1 promotes the formation of lamellipodia and the spreading of growth cones. Rac1 has been first described to directly interact with Plexin-B1 (Vikis et al., 2000). Activation of Plexin-B1 by Sema4D recruits active Rac1 through the RBD domain. Normally, active Rac1 triggers the activation of diverse downstream effectors leading amongst others to reorganization of actin cytoskeleton. Plexin-B1 has been implicated to bind and to sequester active Rac1 away from its effectors and to negatively control the respective pathways (Hu et al., 2001; Vikis et al., 2002). Active Rac1 was also suggested to cluster Plexin-B1 at the cell-surface, thereby functions as upstream of the receptor (Vikis et al., 2002). Various reports have described that Rac1 also contributes to class A Plexin signaling (Jin and Strittmatter, 1997; Kuhn et al., 1999). It has been shown that Rac1 interacts directly but weakly with Plexin-A1 (Turner et al., 2004). Plexin-A1 was found to require active Rac1 for stimulation of cell collapse, whereby Rac1 binding disrupts a potentially autoinhibited conformation of the split GAP domain. Thus, Rac1 was proposed to function as upstream of Plexin-A1 (Turner et al., 2004).

The constitutive active member of the Rho family Rnd1 binds and activates p190 RhoGAP, thus antagonizes the effect of RhoA (Wennerberg et al., 2003). Rnd1 has been reported to bind to Plexin-A1 and promotes its RasGAP activity (Rohm et al., 2000). Rnd1 mediated stimulation of Plexin-A1 has been shown to be blocked by RhoD, which keeps the receptor turned off (Zanata et al., 2002). Rnd1 has been also reported to be involved in binding and regulation of Plexin-B1 (Oinuma et al., 2003), which indicates of the important function of Rnd1 in Plexin signaling.

2.4. Structural basis of Plexin-B1 interaction with Rho proteins

Members of the Rho family have been shown to directly interact with the Rho binding domain of Plexin-B1 (B1RBD), such as Rnd1, Rac1 and RhoD (Bell et al., 2011; Driessens et al., 2001; Tong et al., 2007; Tong et al., 2008; Vikis et al., 2000). NMR experiments showed that all three proteins interact with the same region, β-strands 3 and 4 and a short α-helical segment of B1RBD (Tong et al., 2007). Confirming with this, crystal structures of Plexin-B1 in complex with Rnd1 (Wang et al., 2011) and Rac1 (Bell et al., 2011) showed that the binding interface of the complexes is completely identical (Fig. 8).

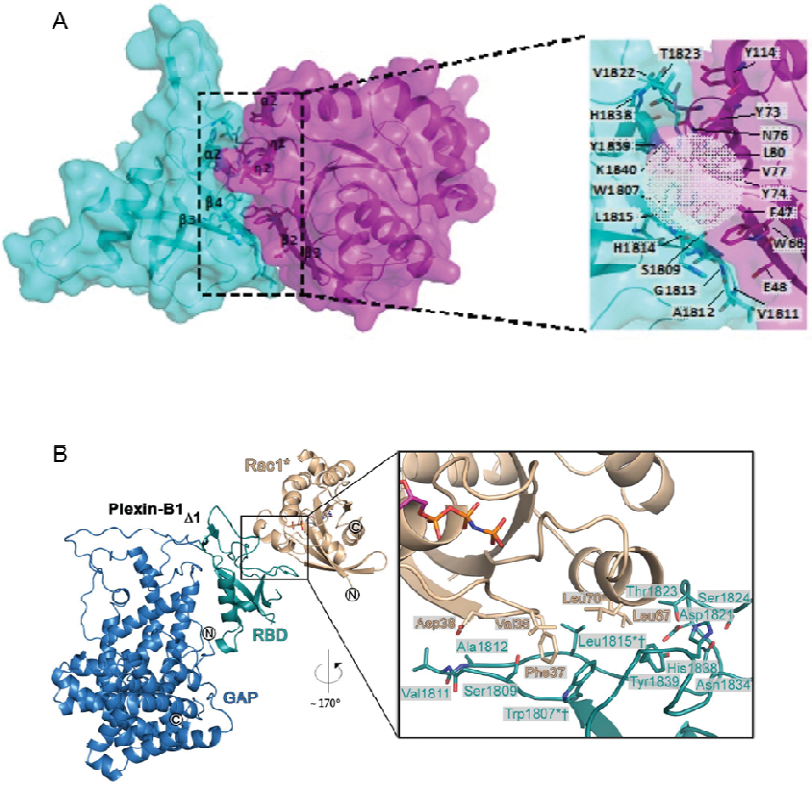

Figure 8: Crystal structure of Plexin-B1 in complex with Rho proteins. (A) Crystal structure of Rho binding domain of Plexin-B1 in complex with Rnd1. Modified from (Wang et al., 2011). (B) Crystal structure of the intracellular domain of Plexin-B1 in complex with active Rac1. Modified from (Bell et al., 2011). Analysis of the binding interface of both complexes confirms that Rnd1 and Rac1 share the same surface of Plexin-B1. Seven hot spots (Ser^{1809}, Gly^{1810}, Gly^{1813}, His^{1814}, Leu^{1815}, Val^{1822}, and Thr^{1823}) on the side of B1RBD are mainly responsible for the interaction with Rnd1 and Rac1. Val^{36}, Phe^{37}, Asp^{38}, Leu^{67} and Leu^{70} (Rac1 numbering) and the corresponding residues of Rnd1 are the hotspots on the side of Rho proteins, which are mainly involved in the interaction with B1RBD. These residues are localized only within the switch regions.

However, the molecular details of the interaction of Plexin-B1 with Rho proteins are not completely clear. It is unknown so far whether Plexin-B1 directly interacts with members of the Rho family other than Rnd1, Rac1 and RhoD. The mechanism, by which B1RBD selectively interacts with distinct protein of the Rho family, is still an important question. How Plexin-B1 discriminates between Rac1 and Rnd1is also an important issue. Investigation of all these aspects would add new insights towards understanding the Rho proteins interactions with RBDs of Plexins, which are a possible activation/inhibition mechanism in context of the function and cell signaling of the full length Plexin receptor. These insights are needed together with the cellular information to design potential agents, which may help in neuronal or cardiovascular regeneration and also in other cellular functions mediated by Plexins. In addition to structural analysis, biophysical and biochemical as well as functional mutational studies are needed to validate the aspects mentioned above.

3. IQGAPs

The members of the IQGAP family of proteins are multidomain scaffolding proteins conserved from yeast to human cells. The multiple domains mediate the binding to target proteins allowing IQGAP proteins to forward extracellular signals. IQGAPs localize to actin-containing structures, such as lamellipodia, phagocytic cups, cell-cell adhesions, membrane ruffles and the actomyosin rings, thus are involved in cell morphology and migratory processes. The IQGAP family proteins comprise three members IQGAP1, IQGAP2 and IQGAP 3, which exhibit strong differences in their tissue distribution. Thus, IQGAP1 is a ubiquitous protein; IQGAP2 is mainly expressed in the liver, stomach and platelet, whereas IQGAP3 was detected mainly in the brain and in the lungs (Schmidt et al., 2003; Wang et al., 2007). IQGAP proteins are evolutionarily conserved and have a very high sequence homology (Fig. 9).

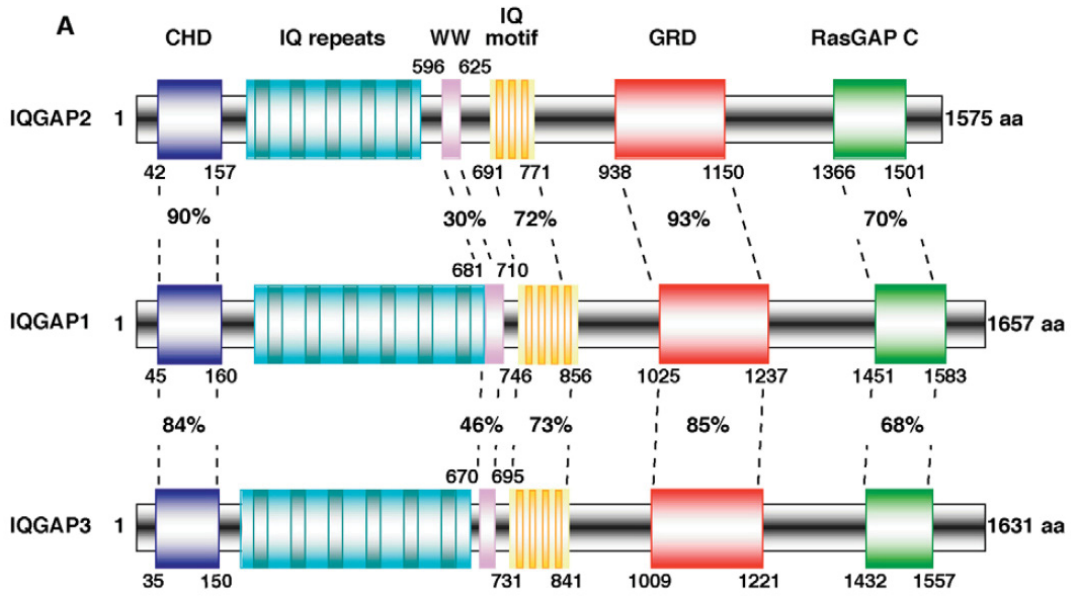

Figure 9: Schematic representation of the IQGAP proteins. IQGAP proteins are multidomain proteins consisting of a calponin-homology domain (CHD), a domain with specific IQGAP repeats (IQ repeats), a proline-rich domain with two conserved tryptophan residues (WW), a domain with three to four IQ motifs (IQ motif), a domain with homology to RasGAPs (GRD, GAP Related Domain) and a C-terminal domain (RasGAP C). Modified from (Wang et al., 2007).

The name "IQGAP" was based on two structural features. Thus, the proteins have calmodulin-binding IQ motifs and a Ras-related GTPase activating proteins (RasGAPs). In contrast to RasGAPs, which accelerate the GTP hydrolysis of Ras proteins, IQGAP proteins exhibit an inhibitory effect on the intrinsic GTPase activity of small GTPases of the Rho family. By interacting with Cdc42 and Rac1 in their active state, IQGAP proteins stabilize Rho proteins in the active form (Hart et al., 1996; Kuroda et al., 1996). The

reason for the lack of GAP function of IQGAP proteins is probably the absence of an evolutionarily conserved arginine finger in their GAP-related domain, which is essentially involved in the catalysis of the GAP activity of RasGAPs (Scheffzek et al., 1998b; Wang et al., 2007).

3.1. IQGAP1 domains and interacting partners

The protein interactions and functions of IQGAP1 have been well investigated, whereas for IQGAP2 and IQGAP3 very less information is known. The 189 kDa protein IQGAP1 is first identified member of the IQGAP family proteins and since then has been extensively studied. The multiple domains of IQGAP1 allow Interactions with many target proteins (Noritake et al., 2005) (Fig. 10).

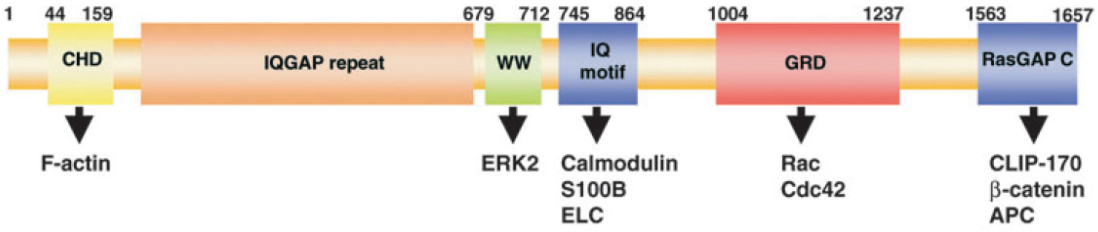

Figure 10: Schematic representation of the protein interaction domains of IQGAP1. The modular structure of IQGAP1 protein and the binding sites of some well-studied binding partners are shown. Calponin-homology domain (CHD), internal repeats (IR), region with two conserved tryptophan residues (WW), four conserved IQ motifs (IQ motif), RasGAP-related domain (GRD) and RasGAP C-terminus (RGCT). Modified from (Noritake et al., 2005).

The N-terminally located domain shares homology with the Ca^{2+} and calmodulin-binding protein calponin (calponin homology domain, CHD) (Winder and Walsh, 1990). Through this domain IQGAP1 interacts weakly with calmodulin and also binds Ca^{2+} ions (Ho et al., 1999). In addition, the CHD domain is responsible for the direct interaction with F-actin (Bashour et al., 1997). No interacting partners have been reported so far for the six specific IQGAP repeats region, which comprise a consensus sequence of eleven amino acid residues (LNEALDEGDAQ). The repeat region is followed by proline-rich region, which contains two conserved tryptophan residues (WW) and is involved in the interaction with the protein kinases ERK1 and ERK2 (Roy et al., 2004). This is followed by the IQ domain, which contains four IQ motifs. The IQ motifs comprise 20-25 amino acids with the consensus sequence IQXXXRGXXXR. These motifs mediate the interaction of IQGAP1 with the EF hand Ca^{2+}-binding protein calmodulin and myosin ELC (Essential Light Chain) and S100B (Hart et al., 1996; Mbele et al., 2002; Weissbach

et al., 1998). Moreover, the IQ is the binding sites for protein kinases MEK1, MEK2 and B-Raf (Ren et al., 2007; Roy et al., 2005). The GRD (RasGAP-related domain), which has homology to RasGAPs, interacts with the Rho proteins Cdc42 and Rac1 (Hart et al., 1996; Kuroda et al., 1996). The RGCT (RasGAP C-terminus) domain at the very C-terminus of IQGAP1 has binding sites for proteins which are involved in cell adhesion, such as E-cadherin and β-catenin (Fukata et al., 1999; Kuroda et al., 1998). Additionally, RGCT domain comprises binding sites for a promoter of actin filament branching N-WASP, the microtubule-associated protein CLIP-170 and the important tumor suppressor protein APC (Fukata et al., 2002; Le Clainche et al., 2007; Watanabe et al., 2004).

3.2. IQGAP1 / Rho proteins signaling interplay

The interaction of IQGAP1 with Rho proteins is involved in actin cytoskeleton reorganization and regulation of cell-cell adhesion. Thus, IQGAP1 binds with its CHD directly to F-actin, which has been shown to increase cross-linking of actin filaments (Bashour et al., 1997). This function is positively regulated by IQGAP1oligomerization and by the binding of active Cdc42 (Fukata et al., 1997).

As an activator of Cdc42 and Rac1, IQGAP1 stimulates the formation of filopodia and lamellipodia of migrating cells (Swart-Mataraza et al., 2002). The overexpression of IQGAP1 enhances migration of cells by increasing actin-rich structures, which are essential for the locomotion of cells (Mataraza et al., 2003). IQGAP1 binds via its C-terminal domain to N-, VE-, and E-cadherin as well as to β-catenin (Kuroda et al., 1998; Schrick et al., 2007; Yamaoka-Tojo et al., 2004), which are essential components of intercellular adherens junctions. In this regards, it was shown that overexpression of the IQGAP1 has inhibitory effect on the E-cadherin-mediated cell-cell adhesion, which results from the binding of IQGAP1 to β-catenin that this can no longer interact with α-catenin, thereby the connection between cadherins and the cortical actin network is blocked (Kuroda et al., 1998). These processes are regulated by Cdc42 and Rac1. It could be shown that the interaction of Cdc42 and Rac1 with IQGAP1 leads to a reduced binding of β-catenin in IQGAP1, which results in stabilization of cell-cell connections (Fukata et al., 1999) (Fig. 11).

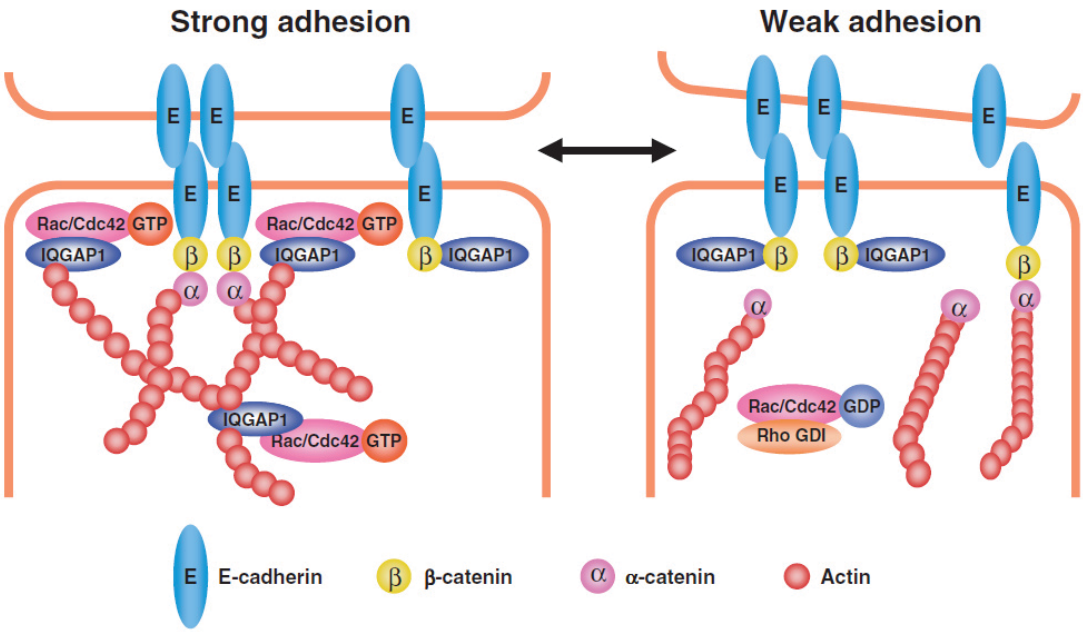

Figure 11: Regulation of cell-cell adhesion by IQGAP1/Rho proteins interaction. In their active (GTP-bound) form, Cdc42 or Rac1 interact with IQGAP1 resulting in the stimulation of actin crosslinking. In this case, E-cadherin is linked with the actin cytoskeleton via β-catenin and α-catenin resulting in a strong cell-cell adhesion. When Rac1 and Cdc42 exist predominantly in the inactive (GDP-bound) form, they dissociate from IQGAP1. IQGAP1 can now interact with β-catenin and displace α-catenin from the cadherin-catenin complex resulting in a decreased cell-cell adhesion. Modified from (Noritake et al., 2005).

3.3. Structural basis of IQGAP1 interaction with Rho proteins

The direct interaction of IQGAP1 with Rac1 and Cdc42 has been investigated using two fragments of the protein. Most of the studies described so far were carried out with a large C-terminal fragment of IQGAP1, which includes residues (864–1657) (Elliott et al., 2012; Li et al., 1999; Owen et al., 2008; Zhang et al., 1998; Zhang et al., 1997). Recent reports have used a fragment based on the GRD of IQGAP1 comprising residues (950–1407) or (962-1345), which exhibit the smallest region of IQGAP1 that is able to bind Cdc42 (Kurella et al., 2009; Owen et al., 2008). Direct comparison of these two fragments has shown that the C-terminal fragment of IQGAP1 exhibit higher affinity towards Rho proteins compared to the GRD based fragments (Owen et al., 2008). Moreover, the idea that IQGAP1 binding may share some similarity with RhoGAP binding to Cdc42 and Rac1 was examined using Ras-RasGAP structure to create a model of the complex between Rac1 and IQGAP1 (Owen et al., 2008) (Fig. 12).

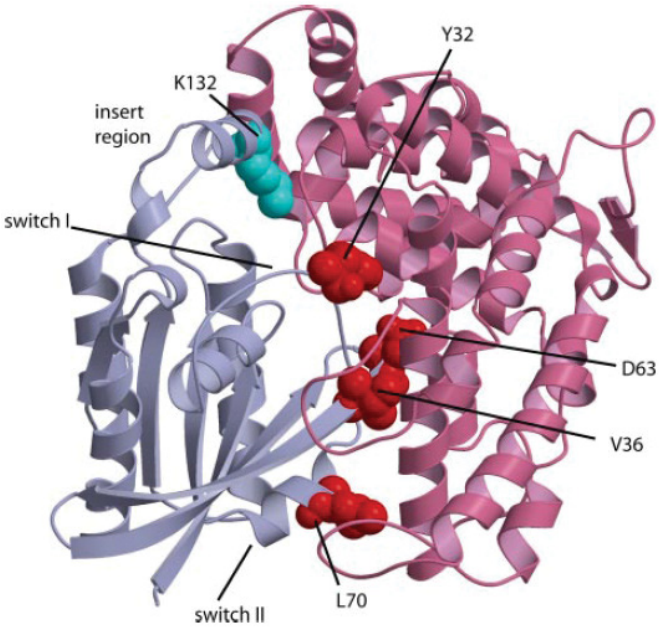

Figure 12: Model of the Rac1-IQGAP1 complex. Rac1 is shown in lilac and IQGAP1 is shown in rose. The model shows that residues within the switch regions and also the insert loop of Rac1 are in close vicinity of the IQGAP1 interface and might be involved in the direct interaction. Modified from (Owen et al., 2008).

However, the molecular mechanism of the interaction of IQGAP1 with Rho proteins is still unknown. Also unclear is whether other members of the Rho family beyond Cdc42 and Rac1 are also able to interact with IQGAP1. How the interaction selectively is mediated, is still an important question. Whether the C-terminal fragment of IQGAP1 achieves additional contacts with Rho proteins or only stabilizes the interaction of the GRD fragment, is also one of the challenging issues in this respect. Structural analysis as well as biophysical and biochemical studies are needed to investigate the above mentioned aspects, which would add new insights towards understanding the structure function relationship of the interactions between IQGAP1 and the Rho proteins. These insights are needed together with the cellular information to be able to understand the regulation of IQGAP1 by Rho proteins, which may lead to discover new tools to study cellular adhesion or design new therapeutic agents against IQGAP1-related diseases such as tumor metastasis.

Chapter 1: New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm*

Jaiswal, M., Fansa, E.K., Dvorsky, R., and Ahmadian, M.R. (2013). Biol Chem *394*, 89-95, doi: 10.1515/hsz-2012-0207.

Background: 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, nucleotide binding, 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. Based on these three classical Rho proteins, it is believed that the intrinsic GTP hydrolysis of Rho proteins is faster than intrinsic GDP/GTP nucleotide exchange. Thus, Rho proteins were understood to persist in the GDP-bound (inactive) form or alternatively in complex with GDIs in resting cells. In this study we utilized fluorescence spectroscopic and HPLC methods as well as recombinant purification of 15 Rho proteins, including RhoA, RhoB, RhoC, Rnd1, Rnd2, Rnd3, Rac1, Rac2, Rac3, RhoG, Cdc42, TC10, TCL, RhoD and Rif, to compare the intrinsic properties all Rho family proteins in one platform.

Results: Comprehensive investigation revealed critical differences among the members of Rho family proteins in their intrinsic properties. Thus, RhoA, Rac1, Rac3 and Cdc42 have strikingly rapid nucleotide association compared especially to that of related RhoB, Rac2 and TC10, which are more than 100-fold slower. The nucleotide dissociation from Rho proteins takes up to 36 h to be completed, demonstrating a strict GEF-dependent Rho protein signaling. Circumstances were different for RhoD and Rif. Nucleotide dissociation from these proteins was relatively fast, exceeding dissociation rate values of other Rho proteins by almost three orders of magnitude. Accordingly, the nucleotide binding affinities of RhoA, Rac1 and Cdc42 proteins are in the high picomolar range, which was 1000-fold lower in the nanomolar range in the case of RhoD and Rif. 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-

* ***Enclosure 1***

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. The Rnd proteins exhibited an extremely low GTP hydrolysis activity but they were able to undergo nucleotide exchange with dissociation rate constants rather comparable to RhoA. Lastly, RhoD and Rif exhibit, similarly to Rac1b, a strikingly faster nucleotide exchange than GTP hydrolysis.

Conclusion: 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 than the intrinsic GDP/GTP exchange. It is generally accepted that such a paradigm for two-state molecular switches depends on the upstream signals such that its equilibrium will be actively shifted to the GTP-bound state. However, this study clearly showed that such a paradigm is not common if we look at the unique features of RhoD and Rif. 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. Moreover, Rnd proteins are not regulated by a conventional GDP/GTP cycling mechanism and accumulate in the GTP-bound form in cells.

Significance: 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, suggesting a shifted paradigm of classical regulation of small GTP binding proteins. Thus, this study is a good starting point for the verification of such hypothesis by conducting more detailed biochemical analysis of Rho family proteins in the interplay with three GDIs, 75 GEFs and 85 GAPs, and their interaction with and activation of more than 100 effector proteins.

Chapter 2: Interaction characteristics of Plexin-B1 with Rho family proteins*

Fansa, E.K., Dvorsky, R., Zhang, S.C., Fiegen, D., and Ahmadian, M.R. (2013). Biochem Biophys Res Commun, doi: 10.1016/j.bbrc.2013.04.012.

Background: Plexin-B1 regulates various cellular processes interacting directly with several Rho proteins. In spite of intense research efforts and advanced knowledge of the biology, biochemistry and structural biology of Plexin-B1 and its interaction with Rho proteins such as Rnd1, Rac1 and RhoD, quite few intriguing molecular details of these interactions remain unclear. It hasn't been reported so far whether other members of the Rho family also have the ability to interact with Plexin-B1. Based on crystal structure of Plexin-B1 in complex with Rac1 or Rnd1, it is believed that Rac1 and Rnd1 bind to the same surface of Plexin-B1. However, how the selectivity of the Rho protein interactions with Plexin-B1 is determined and how Plexin-B1 discriminates between Rac1 and Rnd1, are still important open questions. In this study, we examined *in vitro* and *in silico* the interaction of the Rho binding domain (B1RBD) of human Plexin-B1 with 11 different Rho proteins, including Rac1, Rac2, Rac3, Rnd1, Rnd2, Rnd3, RhoD, RhoA, Cdc42, RhoG and Rif, trying to answer the above mentioned issues.

Results: Valuable findings about the interaction between Plexin-B1 and Rho proteins have been achieved. Thus, B1RBD revealed interaction with the active forms of all Rnd and Rac isoforms as well as with RhoD but not with other members of the Rho family, including RhoA, Cdc42, RhoG and Rif. Competition experiments confirmed that Rnd1 and Rac1 in fact compete for an overlapping binding site on B1RBD and that Rnd1 displaces Rac1 from its complex with B1RBD but not *vice versa*. A C-terminal truncated Rac1 exhibited a significant reduction in the binding affinity for B1RBD. Illumination of the selectivity of Plexin-B1 towards Rho proteins revealed that the differences in B1RBD interacting amino acids, which are analyzed based on the known complex structures with Rac1 and Rnd1, may not *per se* determine the selectivity of the interaction. Instead, B1RBD might utilize additional regions for selective interaction with distinct member of the Rho family proteins. Modeling and functional mutational analysis showed that a

* ***Enclosure 2***

negatively charged loop region, called $B1L^{31}$, might be such a region. Moreover, *in silico* analysis showed that this loop region resides in proximity to the lipid membrane.

Conclusion: Beyond the switch regions, which are most likely the recognition sites of Rho proteins for different interacting partners, additional regions are needed for specifying the selective interaction of Plexin-B1. $B1L^{31}$ loop facilitates to some extent the B1RBD / Rho proteins interaction and thereby might influence the selectivity for distinct Rho protein. This loop region that very likely resides in a close proximity of the lipid membrane may provide a supportive mechanism to regulate the activity of Plexin-B1 in the cellular context. The displacement of Rac1 from Plexin-B1 by Rnd1 supports previous notions that these two proteins might fulfill different requirements in the context of Plexin-B1 signaling. However, such behavior suggests that the binding characteristics of B1RBD to Rho proteins must be different for each particular subgroup. It is tempting to speculate that Rnd1 may have a faster association rate and a slower dissociation rate as compared to Rac1.

Significance: This study added new insights towards understanding the interaction between Rho proteins and the Rho binding domain of Plexin-B1. Plexin RBDs mediate a possible activation / inhibition mechanism in context of the function and cell signaling of the whole receptor. These new findings are needed to understand the regulation of Plexins by Rho proteins in the cellular context, and might lead to development of new tools for investigating the cellular functions mediated by Plexins such as axonal repulsion.

Chapter 3: New insights into the interaction between IQGAP1 and Rho family proteins*

Fansa, E.K., Jaiswal, M., Dvorsky, R., and Ahmadian, M.R.

Background: The scaffolding protein IQGAP1 participates in numerous cellular functions by binding to target proteins, including members of the Rho family proteins. Despite the intensive investigation of IQGAP1 interaction with Rho proteins such as Rac1and Cdc42, major aspects of these interactions remain unstudied. However, kinetic characteristics of these interactions have not been reported so far. Whether IQGAP1 is able to directly interact with other members of the Rho family proteins remains unknown. It is believed that IQGAP1-Cdc42 and IQGAP1-Rac1 complexes differ in the interfaces. Nevertheless, the binding site of IQGAP1 on the surface of Rho proteins is still unknown. Additionally, how the selectivity of the Rho proteins interactions with IQGAP1 is mediated, remains an important open question. In this study, we utilized several biophysical and bioinformatics methods to characterize the interaction of IQGAP1 with 14 different Rho proteins, including Rac1, Rac2, Rac3, RhoG, Rnd1, Rnd2, Rnd3, RhoA, RhoB, RhoC, Cdc42, TC10, RhoD and Rif, in more details.

Results: Novel findings regarding the details of IQGAP1 / Rho proteins interaction have been revealed. Thus, IQGAP1 showed a selective interaction with Rac1- and Cdc42-like proteins, e.g Rac1, Rac2, Rac3, RhoG, Cdc42 and TC10, but not with other members of the Rho family such as RhoA, RhoB, RhoC, Rnd1, Rnd2, Rnd3, RhoD and Rif. In addition, Rac1 and Cdc42 showed an overlapping binding sit on the surface of IQGAP1. Detailed kinetic measurements revealed that Cdc42 displays 3-6 folds higher association rate towards IQGAP1. Cdc42 and Rac2 showed slower dissociation rates with IQGAP1 as compared with other Rho proteins. Equilibrium dissociation constants demonstrated that Cdc42 and Rac2 have the highest affinity to IQGAP1 in the low nanomolar range, while Rac1, Rac3 and RhoG displayed an intermediate affinity in the submicromolar range, and TC10 with the lowest affinity in the micromolar range. Moreover, fluorescence polarization experiments showed that the C-terminal half of IQGAP1 binds with higher affinity to Rac1 and Cdc42 as compared to another fragment comprising only

* ***Manuscript in preparation***

the GAP related domain (GRD) of IQGAP1. Competition experiments utilizing interacting partners of Rac1, e.g. Plexin-B1, $p67^{phox}$, PAK1 and RhoGDIα, along with structural analysis, revealed two areas negative charged areas on the surface of Rho- and Rnd-like proteins, which might explain their inaccessible interaction with IQGAP1.

Conclusion: The overlapping binding site of Cdc42 and Rac1 on the surface of IQGAP1 together with the kinetic details of the selective interaction of IQGAP1 with Rac- and Cdc42-like proteins suggests that these interactions are most likely mediated via the same mechanism. The gain in affinity towards Rho proteins using the C-terminal half of IQGAP1 strongly suggest that the binding interface might extend beyond the GRD domain of IQGAP1, or that the additional regions of the C-terminal half stabilizes the interaction of the GRD domain with Rho proteins. The differences in the overall electrostatic potential of Rho proteins might be an important aspect in defining the selectivity of IQGAP1 for distinct Rho protein.

Significance: This study increased the understanding of the molecular details as well as the structure-function relationship of IQGAP1-Rho protein interactions, which are a key aspect in cell-cell adhesion processes. These new insights are needed to understand the regulation of IQGAP1 by Rho proteins or *vice versa*, and might be helpful to discover new tools to study cellular adhesion mediated by IQGAP1-Rho protein interactions.

Summary

The Rho family proteins were initially characterized as regulators of the cytoskeleton, thereby controlling a diversity of cellular function such as morphogenesis, phagocytosis, pinocytosis, cytokinesis, axonal growth, cell-cell adhesion, cell-substrate adhesion, cell polarity, cell cycle and cell locomotion. Rho proteins function as molecular switches cycling between active (GTP-bound) and inactive (GDP-bound) states. The intrinsic functions of Rho proteins, i.e. GDP/GTP exchange and GTP-hydrolysis are rather slow and require tight regulation. Guanine nucleotide exchange factors (GEFs) catalyze the intrinsic nucleotide exchange, whereas GTPase activating proteins (GAPs) stimulate the intrinsic GTP hydrolysis. Intrinsic functions have been partially investigated in the case of RhoA, Rac1 and Cdc42, but for others not at all. Due to the variety of important cellular processes in which the Rho proteins are involved, the identification and characterization of the target effector has been intensively investigated in order to understand the transmission of signals between Rho proteins and their interacting partners. The interaction of Rho proteins with Plexin-B1, one of the members of the transmembrane Plexin receptors, has been shown to be involved in axonal growth. The scaffolding protein IQGAP1 has been shown to interact with members of the Rho family regulating cell-cell adhesion.

In this thesis a comprehensive analysis of the intrinsic properties of Rho proteins as well as a structure-function analysis of their interaction with Plexin-B1 and IQGAP1 has been described. The analysis of the intrinsic functions of Rho proteins revealed firm evidence for critical differences in the 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. It was believed that the intrinsic GTP hydrolysis of Rho proteins is faster than intrinsic GDP/GTP nucleotide exchange. Thus, Rho proteins were understood to persist in the GDP-bound (inactive) form or alternatively in complex with GDIs in resting cells. The circumstances for RhoD and Rif were different. The nucleotide dissociation from these two proteins was relatively fast, exceeding dissociation rate values of other Rho proteins by almost three orders of magnitude, supporting a shifted the paradigm of classical regulation of small GTP binding proteins.

Valuable findings about the interaction between Plexin-B1 and Rho proteins have been achieved. Thus, B1RBD revealed interaction with the active forms of all Rnd and Rac isoforms as well as with RhoD, but not with other members of the Rho family,

including RhoA, Cdc42, RhoG and Rif. Moreover, Rnd1 was shown to displace Rac1 from its complex with B1RBD. Illumination of the selectivity of Plexin-B1 towards Rho proteins revealed a negative charged loop region, called $B1L^{31}$, which facilitates to some extent the B1RBD / Rho proteins interaction and thereby might influence the selectivity for distinct Rho protein.

Novel findings regarding the molecular details of IQGAP1 / Rho proteins interactions have been revealed. Thus, IQGAP1 showed a selective interaction with Rac1- and Cdc42-like proteins, e.g Rac1, Rac2, Rac3, RhoG, Cdc42 and TC10, but not with other members of the Rho family such as RhoA, RhoB, RhoC, Rnd1, Rnd2, Rnd3, RhoD and Rif. In addition, Rac1 and Cdc42 showed an overlapping binding sit on the surface of IQGAP1. Detailed kinetic measurements revealed that Cdc42 and Rac2 have the highest affinity to IQGAP1 in the low nanomolar range, while Rac1, Rac3 and RhoG displayed an intermediate affinity in the sub micromolar range, and TC10 with the lowest affinity in the micromolar range. Moreover, we showed that the C-terminal half of IQGAP1 binds with higher affinity to Rac1 and Cdc42 as compared to another fragment comprising only the GAP related domain (GRD) of IQGAP1. Lastly we discovered two negative charged areas on the surface of Rho- and Rnd-like proteins, which might explain their inaccessibility and thus their lack of interaction with IQGAP1.

The comprehensive study of the intrinsic biochemical properties of Rho proteins is a good starting point for more detailed biochemical analysis of Rho family proteins in the interplay with three GDIs, 75 GEFs and 85 GAPs, and their interaction with and activation of more than 100 effector proteins. In addition, the structure function analysis of Rho proteins interactions with Plexin-B1 and IQGAP1 increased the understanding of the molecular details and added new insights into these interactions. These new insights are needed together with the cellular information to discover new tools for studying the cellular functions mediated by Plexins and IQGAPs and to design new therapeutic agents against their related diseases.

Zusammenfassung

Proteine der Rho-Familie wurden ursprünglich als Regulatoren der Zytoskelettdynamik beschrieben. Dadurch kontrollieren sie eine Vielfalt zellulärer Funktionen, wie z.B. Morphogenese, Phagozytose, Axonwachstum, Zell-Zell-Adhäsion, Zell-Polarität, und Zell-Migration. Rho-Proteine wirken als molekulare Schalter, die zwischen einem aktiven (GTP-gebundenen) und inaktiven (GDP-gebundenen) Zustand wechseln. Die intrinsischen Funktionen der Rho-Proteine, welche GTP-Hydrolyse und Nukleotid-Austausch umfassen, verlaufen eher langsam und erfordern eine strenge Regulierung. Guanin Nukleotid-Austauschfaktoren (*Guanin nucletide exchange faktors*, GEFs) katalysieren den intrinsischen Nukleotidaustausch, während GTPase-aktivierende Proteine (*GTPase activating proteins*, GAPs) die intrinsische GTP-Hydrolyse stimulieren. Diese intrinsischen Funktionen waren zu Beginn dieser Arbeit im Falle von RhoA, Rac1 und Cdc42 eingehend beschrieben, nicht hingegen für weitere Mitglieder der Rho-Famailie. Durch die Wechselwirkung mit Interaktionpartnern sind Rho-Proteine an einer Vielzahl wichtiger zellulärer Prozesse beteiligt. Die Wechselwirkung von Rho-Proteinen mit Plexin-B1, einem Mitglied der Plexin-Familie von Transmembranrezeptoren, ist in der Regulation des Axon-Wachstums beteiligt. Das *multidomain*-Protein IQGAP1 interagiert mit Mitgliedern der Rho-Familie und ist dabei an der Regulation der Zell-Zell-Adhäsion beteiligt. Im Gegenteil zu klassichen Effektoren von Rho-Proteinen, wie z.B. der Rho-assoziierten-Proteinkinase (*Rho-associated protein kinase*, ROCK), deren Interaktion mit Rho-Proteinen intensiv untersucht und charakterisiert worden ist, waren zu Beginn der Arbeit molekulare einige Details der Interaktionsmechanismen von Rho-Proteinen mit Plexin-B1 und IQGAP1 unbekannt.

In dieser Arbeit wurden eine umfassende Analyse der intrinsischen Eigenschaften von Rho-Proteinen sowie eine Struktur-Funktion-Analyse ihrer Interaktionen mit Plexin-B1 und IQGAP1 durchgeführt. Die Analyse der intrinsischen Funktionen von Rho-Proteinen ergab kritische Unterschiede in deren biochemischen Eigenschaften nicht nur innerhalb der sehr verwandten Rho- und Rac-Isoformen, sondern auch zwischen allen Mitgliedern der Rho-Familie. Es war angenommen, dass die intrinsische GTP-Hydrolyse von Rho-Proteinen schneller verläuft als der intrinsische GDP / GTP Nukleotid-Austausch. Des Weiteren wurde vermutet, dass Rho-Proteine in ruhenden Zellen in der GDP-gebundenen (inaktiven) Form oder alternativ im Komplex mit Guanin-Nukleotid-Dissoziation Inhibitoren (*Guanine nucleotide dissociation inhibitors*, GDIs) vorliegen.

Dagegen, zeigten jedoch RhoD und Rif eine um fast drei Größenordnungen schnellere Nukleotid-Dissoziation als alle bisher untersuchten Rho-Proteine. Diese Befunde unterstützen eine Verschiebung des Paradigmas der klassischen intrinsischen biochemischen Eigenschaften von kleinen GTP-bindende Proteinen.

Im Rahmen dieser Arbeit konnten ebenfalls die Interaktion von Plexin-B1 mit Rho-Proteinen näher charakterisiert werden. Eine Interaktion der Rho-bindenden-Domäne von Plexin-B1 (B1RBD) mit den aktiven Formen aller Rnd- und Rac-Isoformen sowie mit RhoD, nicht aber mit den anderen Mitgliedern der Rho-Familie, konnte *in vitro* demonstriert werden. Darüber hinaus konnte gezeigt werden, dass Rnd1 in einer kompetitiven Art und Weise Rac1 aus seinem Komplex mit B1RBD verdrängen kann. Eine detaillierte Untersuchung der Interaktionsselektivität von Plexin-B1 mit Rho-Proteinen konnte eine negativ-geladene *loop*-Region, genannt $B1L^{31}$, definieren, die die B1RBD-Rho-Proteininteraktion erleichtert und dadurch möglicherweise die Selektivität für ein bestimmtes Rho-Protein determiniert.

Des Weiteren konnten in Rahmen dieser Arbeit neuartige Erkenntnisse über die molekularen Details der Wechselwirkung von IQGAP1 mit Rho-Proteinen gewonnen werden. IQGAP1 zeigte *in vitro* eine selektive Interaktion mit Rac1- und Cdc42-ähnlichen Proteinen, nicht jedoch mit den anderen Mitgliedern der Rho-Familie. Detaillierte kinetische Analysen ergaben, dass Cdc42 und Rac2 die höchste Affinität zu IQGAP1 im niedrig-nanomolaren Bereich aufweisen, während Rac1, Rac3 und RhoG durch eine mittlere Affinität im submikromolaren Bereich und TC10 durch die niedrigste Affinität im mikromolaren Bereich gekennzeichnet sind. Schließlich wurden zwei negativ-geladene Bereiche auf der Oberfläche Rho- und Rnd-ähnlicher Proteine definiert, durch deren elektrostatische Wirkung möglicherweise die fehlende Interaktion mit IQGAP1 erklärt werden könnte.

Die Charakterisierung intrinsischer biochemischer Eigenschaften von Rho-Proteinen stellt einen zentralen Ausgangspunkt für das biochemische Verständnis der Interaktionen von Rho-Proteinen mit ihren Regulatoren und Effektoren dar. Die hier bei der Struktur-Funktion-Analyse der Interaktion von Rho-Proteinen mit Plexin-B1 und IQGAP1 gewonnenen Befunde tragen wesentlich zum molekularen Verständnis dieser Interaktionen bei. Diese neuen *in vitro* Erkenntnisse stellen eine Grundlage für eine weiterführende Untersuchung der zellulären Funktionen von Plexinen und IQGAPs dar, mit dem langfristigen Ziel, therapeutische Ansatzpunkte für assoziierte Erkrankungen zu definieren und zu testen.

Abbreviations

ACK	Cdc42 activated kinase
APC	Adenomatous polyposis coli
CAAX	Cysteine (C)-two aliphatic residues (aa)-variable amino acid (X)
Cdc42	Cell division cycle 42
CHD	Calponin homology domain
Chp	Cdc42 homologous protein
CLIP	CAP-GLY domain containing linker protein
CRIB	Cdc42/Rac Interactive Binding
DBS	Dbl's (Diffuse B-cell lymphoma) big sister
DIA	Diaphanous-related formin
ERK	Extracellular signal-regulated kinases
F-actin	Filamentous actin
Fig	Figure
GAP	GTPase activating protein
GDP	Guanine diphosphate
GDI	GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GRD	GAP-related domain
GTP	Guanine triphosphate
GTPases	Guanosine triphosphatases
GNBPs	Guanine nucleotide binding proteins
HPLC	High-performance liquid chromatography
HVR	Hypervariable region
IQ	Calmodulin binding motif
kDa	Kilo dalton
LARG	Leukemia-associated Rho guanine exchange factor
MEK	Mitogen-activated protein kinase
NLS	Nuclear localization signal
N-WASP	Neural Wiskott-Aldrich syndrome protein
P	Phosphate
PAK1	P21 activated kinase
PKNα	Protein kinase N1

PRG	PDZ (postsynaptic density-95, discs large, and zona occludens) Rho-GEF
PRK1	Actin-regulating kinase
$P67^{phox}$	Neutrophil cytosolic factor 2
Rac1	Ras related C3 botulinum toxin substrate 1
Ras	*Rat sarcoma*
RBD	Rho binding domain
REM	Rho effector homology proteins
Rho	*Ras homolog*
RhoBTB	Rho Broad-Complex, Tramtrack and Bric-a-brac
RKH	ROK-kinectin homology proteins
ROCK	Rho-associated coiled-coil kinase
Sema	Semaphorine
TC10	Teratocarcinoma 10
TCL	TC10-like
WASP	Wiskott-Aldrich syndrome protein
Wrch-1	Wnt-regulated Cdc42 homolog-1

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