Deciphering the molecular and functional basis of RhoGAP family proteins: A systematic approach towards selective inactivation of Rho family proteins

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*Running title: Molecular basis of the RhoGAP family proteins
SUMMARY
Rho GTPase-activating proteins (RhoGAPs) are one of the major classes of regulators of the Rho-related protein family that are crucial in many cellular processes, motility, contractility, growth, differentiation, and development. Using database searches we extracted 66 distinct human RhoGAPs, from which 57 have got a common catalytic domain capable of terminating Rho protein signaling by stimulating the slow intrinsic GTP hydrolysis (GTPase) reaction. The specificity of the majority of the members of RhoGAP family is largely uncharacterized. Here, we comprehensively investigated the sequence-structure-function relationship between RhoGAPs and Rho proteins by combining our in vitro data with in silico data. The activity of 14 representatives of the RhoGAP family towards 12 Rho family proteins was determined in real-time. We identified and structurally verified hotspots in the interface between RhoGAPs and Rho proteins as critical determinants for binding and catalysis. We have found that the RhoGAP domain itself is nonselective and in some cases rather inefficient under cell-free conditions. Thus, we propose that other domains of RhoGAPs confer substrate specificity and fine-tune their catalytic efficiency in cells.

INTRODUCTION
Hydrolysis of the bound GTP to GDP and inorganic phosphate is the timing mechanism that terminates signal transduction of the majority of Rho family proteins returning them to their inactive, GDP-bound state (1,2). The intrinsic GTP hydrolysis (GTPase) reaction is usually very slow (2), but can be stimulated by several orders of magnitude through the interaction of the Rho proteins with Rho GTPase-activating proteins (RhoGAPs) (3-8). RhoGAPs are defined by the presence of a conserved catalytic domain of approximately 190 amino acids, which supplies a conserved arginine residue termed as ‘arginine finger’. This complements an inefficient active site by stabilizing the transition state of the GTPase reaction of the Rho proteins (4,9-14). Most remarkably, the same mechanistic strategy has been shown for bacterial GAPs, such as the Salmonella typhimurium virulence factor SptP, the Pseudomonas aeruginosa cytotoxin ExoS and Yersinia pestis YopE, even though they do not share any sequence or structural similarity with eukaryotic RhoGAP domains (15-17).
Mining in the UniPROT database, we identified 66 distinct human proteins containing a common RhoGAP domain (Fig. 1; Table 1), a number that is slightly different from previous reports (18-25). Among them p50RhoGAP (26), also known as Cdc42GAP (27), p190 (28), and BCR (29) were the first identified and also the best characterized family members. Apart from conserved RhoGAP domains, RhoGAP family proteins possess several sequence motifs and structural domains, which play a role in autoregulation (30), lipid membrane association, subcellular localization and connection to upstream signals (8,18,21,31,32). The majority of RhoGAP family members are largely uncharacterized. To date, the selectivity of about one third of RhoGAPs has been experimentally determined, mainly for their activities towards Cdc42, Rac1, and RhoA using diverse methods (Table 1) (8,18,21,25,31,33,34). Despite their significance, the data reported so far do not allow general conclusions about their selectivity (bimolecular recognition and interaction), efficiency (the capability of GAPs to accelerate GTP hydrolysis), and specificity (multimodal interaction at specific subcellular site influenced by additional domains, motifs and scaffold/adapter proteins) towards Rho proteins, mostly due to a large variation of methods and experimental designs. To revise this status quo, we performed a meta-analysis, aiming to evaluate sequence-structure-function relationship of a variety of RhoGAPs and Rho proteins under cell-free conditions. Therefore, we first measured the GAP activities of 14 RhoGAPs towards 12 GAP-competent Rho proteins and related them to the intrinsic GTP-hydrolysis properties of Rho proteins (35,36) to calculate the fold activation by RhoGAP domain. Second, we combined obtained data with sequence alignments, evolutionary analysis, accessible structural and functional data from previous studies. All information were then systematically assessed in an ensemble approach focusing on various biochemical aspects of the Rho-RhoGAP interactions. Extracted data at the final stage enabled us to predict activity and selectivity of 66 RhoGAPs and to conclude that the specificity of the non-selective GAP domain in cells is most likely
determined by other functional motif(s), and domain(s) in the respective polypeptide chain.

RESULTS

Rho and RhoGAPs: A wide and complex network—Our original intention was to inspect the GAP activity of 14 representative RhoGAPs (Table 1; Fig. S1) toward 14 Rho proteins with GTPase activity (Fig. S2) (35,36). GTPase deficient, GAP insensitive Rho family members, such as Rnd proteins and RhoH/TTF (35), were excluded. As purified Wrch1 and Chp/Wrch2 proteins were not stable in our hand, the following 12 Rho proteins were included in our study: RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, RhoG, Cdc42, TC10, TCL, RhoD, and Rif.

Stimulated GTP hydrolysis reaction was measured by real time fluorescence spectroscopic methods using purified recombinant proteins. In this method, rapid hydrolysis of tamra-GTP by the respective Rho proteins was monitored in the presence of an excess amount of the respective RhoGAPs using a stopped-flow instrument (Fig. 2). Fluorescent tamra-GTP has been previously described as a GTP hydrolysis sensor for most Rho proteins (6). To be able to detect activity of RhoGAPs with even very low efficiency, we used a 50-fold higher molar concentration of the RhoGAP domains above the respective Rho protein, in all GAP-stimulated reactions. However, even such excess did not lead to a measurable GAP activity of some proteins, such as OCRL1 and p85α (data not shown), which were, thus, excluded. Ultimately, p50GAP (hereafter called p50), Oligophrenin 1 (hereafter called OPHN1), GRAF1, Rich1 (also called Nadrin), p190A (hereafter called p190), Abr, MgcRacGAP (also called RacGAP1; hereafter Mgc), DLC1, DLC2, and DLC3 were used in this study.

GAP-stimulated cy3GTP hydrolysis by Rho proteins—The measurement of the GAP stimulated tamra-GTP hydrolysis was suboptimal for RhoA, RhoB, and RhoC (Fig. 2). Therefore, we turned to other fluorescent nucleotides that enable reliable monitoring of real-time kinetics of the hydrolysis reaction by Rho isoforms. We examined the following GTP analogs: ATTO550-GTP, ATTO495-GTP, ATTO488-GTP, FAM5-GTP, and cy3-GTP. As the p50-stimulated cy3-GTP hydrolysis by RhoA provided a substantially distinct decrease in fluorescence (Fig. 2), we measured and evaluated the RhoGAP activities towards RhoA, RhoB, and RhoC using cy3GTP.

In total, we have measured hydrolysis of fluorescently labeled GTP by 12 Rho proteins in all mutual combinations with 10 RhoGAPs. Evaluated observed rate constants (k_\text{obs}) are shown in Figure 3 as a bar diagram and summarized in Table 2 in fold activation. Intrinsic GTP hydrolysis was used as control experiments.

RhoGAPs lack selectivity—In general, the investigated GAPs do not show any selectivity towards particular Rho proteins or their isoforms. p50 appears to be most universal GAP as it stimulated the GTP hydrolysis of all Rho proteins more than 200-fold as compared to the intrinsic GTPase reaction (Fig. 3). However, there is a large difference between the highest activity towards Cdc42 with k_\text{obs} value of 14 sec^{-1} and the lowest activity towards RhoD with k_\text{obs} value of 0.12 sec^{-1} (Fig. 3). p190 was also highly active on all Rho proteins with a relatively low stimulation of the Rif GTPase reaction. Noteworthy, p190 revealed a high activity towards RhoD that was as efficient as p190 activity towards RhoA.

OPHN1, a Bin/Amphiphysin/Rvs (BAR) domain-containing protein (see Fig. 1) exhibited overall highest activities reaching a stimulation of 5 orders of magnitude in the case of RhoA and RhoB. GRAF1, an OPHN1-homologous protein, was found with an absolute k_\text{obs} value of 90 sec^{-1} for Cdc42 as a fastest stimulated GTP hydrolysis reaction among the GAPs investigated in this study. Differences between fastest and slowest stimulation for both, OPHN1 or GRAF1, remarkably exceed 3 orders of magnitude, pointing to an extreme span of measured activities for a single GAP. Intermediate activities were measured for Mgc, Rich1 and Abr, which are still able to stimulate the GTP hydrolysis of measured Rho proteins but to a significantly lower extend than previously mentioned RhoGAPs, especially for TCL and Rho isoforms. Accordingly, all three proteins can be classified as preferential to Cdc42 and Rac isoforms, with an addition of Mgc acting on RhoD and Rich1 acting on TC10. Rather inefficient GAP activities were detected for the DLC isoforms, except for the DLC1 activity towards the Rho isoforms and marginally towards Cdc42, Rac1, and TC10. Overall disability of DLC2 and DLC3 proteins...
to operate on analyzed Rho proteins raises a question about proper conditions under which these proteins may exert their GAP functions.

New insights from differential catalytic efficiencies—A remarkable finding of our analysis is a broad spectrum of catalytic efficiencies and substrate-selective properties of investigated proteins ranging from a 1-fold to a 120,000-fold stimulation of the intrinsic GTP hydrolysis (Table 2). To illustrate this explicitly, we plotted all 120 pairs of RhoGAP and Rho proteins (x-axis) against fold activation (y-axis) in numeric order starting with OPHN1-RhoB with the highest efficiency and ending with DLC2-RhoG with no activity (Fig. 4; Table 2). Overall, the RhoGAP-Rho protein pairs were subdivided into six groups based on their catalytic efficiency to stimulate the intrinsic GTP hydrolysis of the Rho proteins (Fig. 4). OPHN1 and its homolog GRAF1 form the first group as they have emerged as highly efficient GAPs not only for the Rho isoforms but also for Cdc42 and TC10 (Fig. 4). p50 and p190 also belong to this group GAPs with the highest catalytic efficiency particularly for their selectivity towards RhoB and RhoD, respectively. Mgc and Rich1 rank to the second high efficiency group for their activities towards Rac1, RhoD, and TC10. We also indexed p190 to this group as it clearly revealed significantly high activities towards Rac1 and Rac3. The third group with intermediate efficiency is interestingly populated by Rho isoform-specific DLC1 and Rac-specific Abr. The p50-Rif pair is the most active in the fourth group which overall displays low efficiency. However, as p50 showed a poor selectivity (Table 2) it is rather doubtful that p50 may be a physiological RifGAP. Caution should be applied when looking at the data of group five (with 45 pairs the largest group) in Figure 4 (pairs between 10- and 150-fold activations). To this group belong protein pairs with lowest activity, for instance the DLC isoforms on the one hand, and Rif and RhoD on the other. We scored this group despite their obvious but low GAP activities as inefficient pairs. A very small group of only 4 pairs with an output of less than 10-fold activation was graded as ‘inactive’ due to their extremely low catalytic efficiency.

The two critical steps in stimulating GTP hydrolysis—Two critical steps that may control the catalytic efficiency of the GAPs under the conditions used in this study are the (i) association of the RhoGAP with the GTP-bound Rho protein, and (ii) the stimulation of the GTP hydrolysis reaction itself. To examine whether an association-controlled mechanism is a reason for the extreme differences in the catalytic efficiency, we loaded Cdc42 with tamra-GppNHp, a non-hydrolysable fluorescent GTP analog (6), and measured in real-time its association with the RhoGAPs. As shown in Figure 5, there is a clear correlation between the reaction rates of association and GTP hydrolysis. Correspondingly, DLC isoforms, for example, revealed a 1500-fold lower association rate when compared to OPHN1 (Fig. 5B). These data strongly suggest that the catalytic efficiency of the RhoGAPs is directly proportional to the rate of their association with the Rho proteins.

Identification of hotspots within protein interfaces—To inspect molecular details of interaction between Rho proteins and RhoGAPs we have analyzed 42 structures of RhoGAP alone and in the complex with Rho proteins available in the PDB (Table S1). Residues involved in intermolecular interaction were defined to have at least one inter-atomic distance shorter than 4.0 Å. They constitute interacting interface highlighted on the crystal structure of RhoA-p50GAP (Fig. 6A). We have extracted information about interacting amino acids from different complex structures and combined them with sequence alignments of all investigated proteins in the form of an interaction matrix (Fig. 6B). Each element of the matrix, that we call ‘hotspot’, relates one homologous residue from Rho proteins to one homologous residue from RhoGAPs (see also Figs. S1 and S2). The number value of this element represents the number of complex structures in which these residues interact. Thus, a zero value of the element means that these two residues do not face each other in any structure while the value 8 means that this particular interaction is to be found in all known structures. We have sorted the residues at both sides of the matrix according to the conservation vs. variability. As can be seen (Fig. 6B), more than a half of the residues (17 out of 24) on the side of Rho proteins are identical or highly conserved (G/A15, S/T37,
D/E64, D/E65). These residues comprise mostly switch I and switch II and create a continuous patch on the surface (Fig. 6A, left panel). On the other side, only 6 amino acids are identical in RhoGAPs, 5 are homologous, and the majority of the GTPase interacting residues are variable (Fig. 6A, right panel). Strikingly, identical and conserved residues form a patch on the GAP domain. We postulate that the interaction between conserved patches of Rho proteins and RhoGAPs is responsible for both the recognition of the two proteins and the catalysis of GTP hydrolysis. This hypothesis is supported by the fact that an identical arginine 282 (p50 numbering), known as the arginine finger, essential for the catalysis (12,13,37), is a central residue of the conserved patch on GAP and contacts only identical residues on Rho proteins (Fig. 6). Interactions on this region are not expected to contribute to the differences in activities (or selectivities) and were, thus, excluded from further analysis. However, the number of remaining variable pairs is still high and indicates that the relationship between observed diversity of stimulation and molecular interactions is not simple but quite complex and multifaceted.

The four most effective GAPs, including OPHN1, GRAF1, p190, and p50, share an asparagine (glutamine in p190) at position 417 (p50 numbering), which is not present in other investigated GAP domains (Fig. 6B). Their predominant counterpart residue in Rho proteins is Tyr-66. Its particular interaction with amide group may contribute to higher activity. Moreover, OPHN1, the most efficient RhoGAP (Fig. 4; Table 2), has in this region two unique residues, containing hydroxyl group (Thr-283 and Ser-286; Fig. S1). Thr-283 undergoes a favorable contact with serine and asparagine at the position 88 as well as variable residues at the position 90 of Rho proteins (Fig. 6B). Rac isoforms have at the latter position hydrophobic residues that are disfavored by Thr-283 in OPHN1, which contributes to the lower OPHN1-stimulated GTP hydrolysis by Rac isoforms. Going beyond the variable regions of interacting interface, three of four most active GAPs have unique leucine at the position 386, which is otherwise replaced by a lysine or a glutamine (Fig. 6B). Its counterpart residue on the side of Rho proteins is the invariant Tyr-34. The nature of the interactions, in which they are involved, is contributing to observed differences. Tyrosine can be involved either in hydrophobic interactions with leucines by utilizing its phenyl moiety or in electrostatic interactions with lysines employing its hydroxyl group. Similarly, DLC isoforms that were found to be least efficient in stimulating the GTP hydrolysis of Rho proteins contain within the interacting interface unique positively charged residues, arginine and lysine at positions 409 and 413, respectively (Fig. 6B). Such amino acids at these positions are rather unfavorable because the presence of prevalently hydrophobic residues is required in this region of the GAP surface as it contacts the hydrophobic-patch on Rho proteins formed by invariant V36, F37, L67 and L70 (RhoA numbering; Fig. 6). However, considering only DLC GAPs, there are no differences in their interacting residues that could explain partial selectivity of DLC1 for the Rho isoforms. Although it is also not directly possible to interpret an overall low activity of RhoGAPs on RhoG, RhoD, and especially Rif, the interaction matrix enables to determine the regions that are very likely responsible for these low activities. They comprise variable positions on Rho proteins, e.g., 90, 97 and 134, and on RhoGAPs, e.g., 283, 286, 287, 288, and 309 (Fig. 6B). A similar situation exists for a considerable effect of p190 on RhoD. RhoD has a unique threonine at position 35 but it interacts with identical residues of GAPs (Fig. 6B). On the other side, p190 has also unique amino acids at positions 323, 390, 408 and 413 but they interact reciprocally with identical residues of Rho proteins. Both proteins interact further through variable regions, thus, a full elucidation of broad spectrum of GAP catalytic activities on Rho proteins would require global evaluation of synergic effect of multifaceted interaction between varying amino acids.

RhoA-p190 complex: crystal structure verified a matrix-based predicted interaction—Taking into account that Rho proteins and RhoGAPs are highly homologous, it is legitimate to assume that yet unknown complex structures will share the same structural architecture. Consequently, corresponding residues according to sequence alignments are expected to interact in the manner of known complex structures. Thus, in the absence of structural information for some Rho-RhoGAP complexes,
interaction matrix enables us to deduce which amino acids could be involved in the interaction between these two proteins. In order to prove the validity of such hypothesis, we solved the crystal structure of RhoA in complex with the GAP domain of p190 at high resolution (Table S3; Fig. 7A) and used this structure as a benchmark for the verification of our assumptions.

As expected, overall structure arrangement of RhoA-p190-GAP complex is similar to complexes of Rho proteins with other GAPs (similarity of p190 to p50 and GAP20 is 25.1% and 17.9%, respectively; Table S2). The RhoA structure corresponds to an active, GTP-bound conformation and clearly differs from its GDP-bound form (Fig. 7A). Conformation of p190 differs in some regions from the structures of p50 and ARHGAP20 (Fig. 7B). However, most relevant for our study were both its high structural similarity in the conserved region of the interacting interface and its conformational variability within the loop between residues 1406 and 1419 (Fig. 7C). Position and orientation of catalytic Arg-1284 are very similar to those found for the arginine fingers in other complexes (Table S1). We note that the weak electron density around the residues 26-31 of RhoA did not allow us to fully build its switch I region.

Relating the complex structure to interaction matrix, nonzero matrix elements can predict possible interactions in RhoA-p190 complex. Higher number of the element indicates higher probability of contact occurrence in complex (Fig. 6B). We have thus calculated interaction matrix only for RhoA-p190 complex structure and compare it with original interaction matrix. Vast majority of conserved residues, which were predicted to be in the contacts, are indeed presented in the structure (Fig. 7). One exception is a conserved arginine at position 323 (Fig. 6B; p50 numbering), which is exclusively a serine in p190A (Ser-1326). These residues are supposed to interact with Glu-65 of RhoA. Structure of the RhoA-p190 complex revealed that Ser-1326 at this site is in the vicinity of Glu-65 but simply not long enough to form the contact with it (data not shown). Largest discrepancies between predicted and observed contacts comprise the interaction of ‘conserved’ patches of Rho proteins and ‘variable 1’ of RhoGAPs. The reason is that sequence alignment of all RhoGAPs (Fig. S1) contains in this region many gaps and shifts which preclude reliable prediction of its structure and proper assignment of similar residues. However, it has to be borne in mind that the interaction matrix was constructed on the basis of only 4 crystal structures. Including more structures for its calculation would certainly increase its reliability and enable more precise prediction of unknown complexes between Rho proteins and RhoGAPs.

p190 acts on RhoA but not on RhoD in cells—An obviously demanding question is, to what extent the substrate selectivity of the RhoGAP domains determined in this study under cell-free conditions reflects and is relevant to that of a multicomponent and multidomains cellular machineries. Addressing this question is of an ultimate importance due to the fact that RhoGAPs did not in general reveal strong selectivity for some particular Rho proteins (Fig. 3, Table 2). An interesting observation in this regard is the high activity of p190 towards RhoD that is comparable to its activity towards RhoA (Fig. 4, Table 2). To answer the question, whether the p190 is also a GAP for RhoD in cells, we used GST-fusion of mDia1-RBD and Rhotekin-RBD to pull down GTP-bound RhoD and RhoA, respectively (38). Strikingly, the obtained data revealed that the amount of pulled down RhoD-GTP remained unchanged in cells overexpressing either full-length (FL) or the GAP domain of p190 (Figs. 8A and 8B). Control experiments showed that p190-FL indeed acts as a GAP for RhoA but overexpression of only the GAP domain failed to stimulate GTPase activity of RhoA (Figs. 8C and 8D). These results provide a clear indication that other domains and motifs of p190-FL determine its specificity for RhoA in cells and not for example for RhoD. Extrapolating it on the activities of the GAP domains measured under cell-free conditions for members of the Rho family we postulate that they are not directly proportional to specificities of GAP proteins within the cell.

The role of other domains as the determinants of GAP specificity—Results described above brought us to the question about the activity of GAP domains in the context of the full-length proteins and their niche within the cell. Another question in the same context is whether and to what extend GAPs can regulate multiple signaling pathways, which in turn
seem to be dominated by the composition of their domains as illustrated with our data. Therefore, we conducted a phylogenetic analysis of 66 human RhoGAP proteins (Table 1) based only on the sequence of respective domains of the RhoGAP family members. The phylogenetic order correlated with the arrangement of proteins according to their domain and motif compositions (Fig. 1). We assigned so far 33 different domains with different properties (Table S4). The majority of them can be classified into the three major groups: (i) lipid and membrane binding domains; (ii) peptide and protein interacting domains, (iii) catalytic domains with enzyme activities (Fig. 1). Most widespread domains are PH (30), CC (25), P (16), SH3 (15), and BAR/F-BAR (14). Most RhoGAPs have 3-4 additional domains, while Cnt-d1 has another 10 and MYO9B even 11 domains (Fig. 1, Tables S4 and S5). Thirteen GAPs lack any additional putative domains but contain highly variable regions at their N- and C-termini. It is possible that these regions consist of not yet identified motifs, which may contribute to their specific function in the cell. ARHGAP11B is the smallest RhoGAP protein belonging to this group (Fig. 1; Table 1). A blast search of the terminal 63 and 17 amino acids of ARHGAP11B revealed a consensus motif, KLL(X5)RED, at its C-terminal region, which exist in many proteins (Data not shown). Both KLL and RED motifs have been reported to be involved in protein-protein interactions (39-41).

DISCUSSION

General Profile: The Rho protein selectivity of the GAP-domain—The most efficient activator of the GAPase reaction among investigated GAP domains is OPHN1 that stimulated GTP hydrolysis of RhoA and RhoB upto 5 orders of magnitude as compared to the other investigated RhoGAPs. Its second striking feature is that it can efficiently deactivate a broad spectrum of investigated Rho proteins, including the Rho and Rac isoforms, Cdc42, TC10, and TCL (Table 2). The ability to stimulate efficiently GTP hydrolysis of various Rho proteins was also observed for GRAF1, p190, p50. These four GAPs are in general active on the same Rho proteins. Least susceptible are RhoG and Rif that could be in fact deactivated only by p50.

While the GTP hydrolysis of RhoG was to a limited extent stimulated by OPHN1 and p190, there is no GAP in our set that could actually act on Rif. Remarkably, GTP hydrolysis of RhoD was only markedly stimulated by p190 and Mgc. The spectrum of activity for Mgc on the different Rho proteins is not as broad as the previously mentioned GAPs. Its activity on Rho isoforms is about 10-fold lower compared to RhoD, Cdc42, and Rac isoforms and it is practically inactive on TC10 and TCL. Rich1 was found to be even less effective, as it significantly stimulated GTP hydrolysis reaction of only TC10 and to a lesser extent of Cdc42, Rac1, and Rac2. Interestingly, TCL, RhoG and Rho isoforms are less sensitive to hydrolysis by Rich1 but on the other hand, Rho isoforms appeared to be exclusive substrate for DLC1. In fact, a unique selectivity was observed for DLC1 GAP domain, which acts specifically on RhoB, RhoA, and RhoC (Table 2). Abr was the least effective but effectively deactivated Rac1 and Rac3. However, we do not designate it as selective for these Rac isoforms because its hydrolytic activity towards other GTPases was not so distinct as the activity of DLC1 for Rho. DLC2 and DLC3 did not show any considerable GAP activity, an observation that raises the question if these proteins still can be considered as RhoGAPs.

Taken together, there is no assured selectivity between investigated RhoGAP domains and particular Rho proteins or their isoforms. Our observation regarding the selectivity of GAP domains is in contrast to the guanine exchange factors (GEFs) of the Dbl family that activate Rho proteins by accelerating the GDP/GTP exchange reaction. We showed in previous study that their isolated DH domain (diffuse B-cell lymphoma (Dbl) homology), which is actually responsible for nucleotide exchange, showed both selectivity and specificity for their substrate Rho proteins (36). This finding challenges fundamental principles of cell signaling exemplifying that there are two principally different manners of interplay between the Rho proteins and their regulatory proteins. In the first case of Dbl family GEFs, the catalytic DH domain directly interacting with the substrate Rho protein is itself able to selectively discriminate among the Rho proteins, and other additional domains and motifs of the full-length RhoGEF protein provide an additional degree of regulation in
the cell. In the second case, when the catalytic domains directly interacting with the substrate Rho proteins do not show any distinct selectivity, as we have found for the RhoGAP domains, secondary domains and motifs of the full-length RhoGAP inevitably determine, beyond other features, the specificity for the substrate Rho proteins. This is nicely demonstrated in this study by an example of p190 protein. Its GAP domain was equally and highly active on RhoA and RhoD under cell-free conditions but its full-length version in the cells was able to specifically inactivate only RhoA and not RhoD. Existence of multiple determinants in full-length RhoGAPs which dictate their localized recruitment, activation, ‘specific’ function in cells by including distinct protein and lipid interaction domains and motifs, as well as post-translational modification has been suggested by several previous studies (18,42-47). It has been shown that the spatial distribution of RhoGAPs and their specificity toward individual Rho proteins are controlled by their interactions with various proteins within signaling complexes (48,49). Our results, thus, elegantly complement the scenario for the function of GAP proteins in which a concerted action of the whole protein is required. Accordingly, we conclude that p190 cannot be recruited to RhoD because it is RhoA specific or p190 and RhoD do not find each other under the used experimental conditions. We can, however, not exclude the possibility that p190 might specifically operate on RhoD in a specific cell type or stimulus.

Pair interaction and interaction matrix—In order to shed light on the molecular interactions of RhoGAPs with Rho proteins, we have analyzed available crystal structures of their complexes and combined the data about interacting residues with two multiple sequence alignments of investigated RhoGAPs and Rho proteins in the form of a structure-based interaction matrix (Fig. 6A). Such interaction matrix allows to predict, which residues of two sets of homologous proteins are likely to interact in their binary complexes. In addition, it provides a complete overview of the occurrence of particular contacts in analyzed structures as well as the conservation or variability of respective amino acids utilized by both GAP domain of RhoGAP proteins and G domain of Rho proteins upon interaction.

In terms of conservation, GAP side residues are largely variable (Fig. S2) in contrast to the Rho side residues, which are mostly identical. The variability of the latter originates almost exclusively from the helix 3 and the insert helix (Figs. 6 and S2). Reordering of residues in the matrix according to their conservation enabled us to divide hotspots into three distinct regions that also correspond to three exclusive regions on interacting interface. Each of these regions includes also distinctive interacting pairs of amino acids (Figs. 6, S1-S3; color-coded regions). They can be classified into three different groups: interaction pairs of conserved residues, pairs of variable residues on both sides and the interactions of conserved residues on Rho side and variable residues on GAP side.

We have hoped that our analysis would reveal special distinctiveness in the interactions between Rho and RhoGAP proteins that could at least semi-quantitatively explain differences in observed activities. What we have found instead was an abundance of combinatorial possibilities and the complexity incorporated in the formation of binary protein complexes. In order to relate observed stimulations of GTP hydrolysis with sequence differences among investigated proteins and to describe them quantitatively, contributions from all matrix points have to be considered. Each element in the matrix represents in principle the combination of 12 Rho protein and 10 RhoGAPs, e.g., 120 possibilities. To assess the contributions of all such combinations requires the evaluation of the impact of all different amino acids at each particular spot. For example, in case of invariant Tyr-34 of Rho proteins all types of its interactions with leucine, lysine or glutamine of GAPs at position 386 have to be considered (Figs. 6B and S3). As the chemical properties of these amino acids are very diverse, so is the nature of their interactions. The situation is even more complex for the second discussed invariant Tyr-66 in Rho proteins because it contacts 9 different amino acids of RhoGAPs (Fig. 6B). Spots that have variable residues on both sides of the matrix would require even more thorough evaluation. Finally, an overall contribution of all individual elements from the interaction matrix would have to be correlated with observed differences in activities in order to obtain fully qualitative description.
Predicted RhoA-p190 interacting interface verified by the crystal structure—However, interaction matrix also allows to predict which residues of one Rho protein would interact with one of RhoGAP. In order to validate this approach we have solved the structure of RhoA-p190GAP complex (Fig. 7A), calculated the interaction matrix exclusively for this structure and compared it with original interaction matrix (Fig. 7C). As can be seen, a majority of residues interacting between conserved patches could be successfully anticipated. Most of deviated contacts pertains the interactions between conserved Rho residues and `variable 1´ of RhoGAPs. Reason for it is gaps in the sequence alignment of GAP domains, namely in hypervariable regions (Fig. S1). They enable shifts of corresponding amino acids so their space positions in the presumed complex structures might differ from positions found in known structures. A comparison of three distinct RhoGAPs from complex structures (Table S1), i.e. p50, RAGAP20 and p190, nicely demonstrates that the conformation of this variable region is indeed very diverse. The whole loop in p190 comprising residues 1406 to 1419 is folded completely differently when compared to the corresponding loop of p50 respectively (Fig. 7B). Interaction of this region of RhoGAPs with conserved residues of Rho proteins seems to be responsible for observed differences in their activities. On the other hand, interaction of conserved patches on both sides of complexes is preserved and exclusively determines the formation of the complex between Rho proteins and RhoGAPs.

Cellular context: How the RhoGAPs determine and regulate specificity?—Several cell-based studies have been shown that there is specificity between RhoGAP and Rho proteins, e.g., ARHGAP15, Ber, β2 chimerin, 3BP1, p68RacGAP and FilGAP are specific for Rac1 (29,50-54), RalBP1 and MgcRacGAP1 for both Cdc42 and Rac1 (55-57), Rich1, CdGAP for Cdc42 (58,59); ARHGAP6, DLC1, DLC3, myosin IXb, OPHN1, p190A and RA-RhoGAP for RhoA (34,47,60-68), ARHGAP18, ARHGAP21 and DLC1 for RhoC (64,66,69), TCGAP for TC10 (70), PARG1 for RhoA (71), and ARHGAP30 for Wrch1 (72).

Our observations, however, show that RhoGAP domains are not able to selectively deactivate a particular Rho protein or its isoforms. Such discrepancy raises the question of what other factors, processes or circumstances may determine the specificity between RhoGAPs and Rho proteins. Cellular context is certainly crucial, as for example studies on p190A (73,74) and Myosin IXb (75) showed that these proteins have different specificity in vitro and in vivo. There are several possibilities for the regulation of the GAP activity in cells ranging from intermolecular autoinhibition (β-chimerin, p50, OPHN1, DLC1) (8,30,76,77) to post-translational modifications (78). For instance, ‘activating’ phosphorylation generates in p190 a new contact site for p120RasGAP (79), releases DLC1 from its autoinhibited state (76), or converts MgcGAP to a Rho-specific GAP (80). Furthermore, SUMOylation of ARHGAP21 may represent a way of guiding its function (81) and non-proteolytic ubiquitination of p250GAP controls axon growth (82).

The fact that almost all GAP proteins consist of several diverse domains and motifs strongly indicates that the regions accompanying the GAP domain are crucial for their function. 66 GAPs identified in human genome contain 33 different domains (Fig. 1; Tables S4 and S5). Some of them possess up to 5 different domains and there are some proteins, which contain 4 or even 5 copies of the same domain (Fig. 1; Table S5). Domain composition of GAPs together with the nature of individual domains demarcates on a higher level their subcellular localization and function. For example, the BAR domain of OPHN1 and GRAF1 is simultaneously involved in membrane tubulation and GAP inhibitory functions (8). The SEC14-like domain of p50 homology appears not only to regulate the GAP activity (77) but also to localize p50 in the endosomal membrane as a link between Rho and Rab proteins (83). Similarly, phospholipid binding to the C1 domain both recruits β-chimaerin to the plasma membrane and activates its RacGAP activity (30). The RhoGAP activity of DLC1 has been proposed to be inhibited by an intramolecular interaction between the SAM and RhoGAP domains (84). Phosphorylation by CDK5 and association with both phospholipids and the scaffold proteins tensin and talin has been shown to...
release DLC1 from its inhibited state and to promote significantly promote its RhoGAP activity (67,76,84,85). C2 or PH domains of GRAF, Abr, OPHN1, and Mgc are the modules mediating association with the membrane according to the calcium-dependent phospholipid binding or phosphatidylinositol concentration (8,31,80,86-90).

Being the smallest human RhoGAP, ARHGAP11B is not decorated with any known domain or motif (Fig. 1). The involvement of ARHGAP11B in neuronal development by promoting basal progenitor amplification and neocortex expansion has been reported recently (91). This study has shown that ARHGAP11B does not exhibit RhoGAP activity as compared to ARHGAP11A and its variants. Instead of directly measuring the GAP activity, however, more downstream RhoA/ROCK activity was assayed. ARHGAP11A and 11B share 90% identical sequences in their RhoGAP domain and mainly differ at the very C-terminal end (91), which is highly variable in all RhoGAPs (Fig. S1). Other residues that are essential for the RhoGAP activity are highly conserved in ARHGAP11B indicating that this GAP, although very small, may act as GAP for Rho proteins. In this context, the C-terminal KLL and RED motifs that were detected in a blast search in this study (see above) may play a role in protein-protein interactions (39-41).

Functionalization of the RhoGAPs with various modular building blocks, especially the membrane-associating domains, is a prerequisite for successful orchestration of a series of spatiotemporal events, including recruitment, subcellular localization, assembly of proactive protein complexes, and ultimately association with and inactivation of the substrate Rho protein. Reduced dimensionality on distinct regions of the cell membrane does not only achieve high specificity of the RhoGAPs but also tremendously enhance their overall catalytic activity.

Enhancing an inefficient GAP or using it for very slow processes—The efficiency of a RhoGAP depends largely on the cellular process, in which they are involved. There are very fast processes, e.g., calcium fluxes, exocytosis or muscle contraction, and very slow process, e.g., differentiation, apoptosis or metabolism, which are also very much dependent on cell types. The GAP protein that is inefficient under cell-free conditions may efficiently operate through the function of its other domains in an appropriate cellular niche. An appropriate example is provided by DLC proteins that were mostly found as inefficient or even inactive in this study (Fig. 4). DLC1 has been thought to play major role as a tumor suppressor in a GAP domain-independent manner (92). However, the DLC1 activity is, as compared to DLC2 and DLC3 activities, relatively high towards the Rho isoforms, Rac1, Cdc42, and TC10 (Fig. 4). Thus, it is conceivable that additional mechanisms contribute to further enhancement of its GAP activity, comprising CDK5 phosphorylation, association with scaffold proteins, such as tensin and talin, and/or association with lipid membranes (67,76,84,85). On the other hand, there are also mechanisms to inhibit the DLC1 RhoGAP activity, including phosphorylation by protein kinases C and D, and subsequent association with 14-3-3 proteins (62) or direct association of the SH3 domain of p120 RasGAP with its RhoGAP domain (32,93). Similar regulatory mechanisms has been proposed for DLC2 and DLC3 (67) suggesting that inefficient RhoGAPs under cell-free conditions can be highly efficient in proper cellular context and appropriate protein network.

An inefficient GAP can otherwise be employed in the control of a slow cellular process, including actin dynamics. A group of nonconventional Rho proteins, such as RhoD, Rif, and Rac1b mainly persists in their active state under resting conditions (35,94). They accumulate in their GTP-bound state and thus are essentially dependent on a specific GAP to be switched off (35). Both RhoD and Rif are involved in the integration of cytoskeletal reorganization and membrane trafficking (95,96), however, specific RhoGAPs for these atypical members of the Rho family remain to be found.

Not all RhoGAP domain-containing proteins are GAPs—According to the mechanism of the GAP-stimulated GTPase reactions, the RhoGAP domain supplies an arginine finger directly into the active site of the substrate Rho proteins to stabilize the transition state (13,97). A first inspection of the sequence alignment of the 66 RhoGAP domains revealed that ARHGAP36, Cnt-d1, DEP1, DEP2, FAM13B, INPP5P, and OCRL1 lack an arginine finger at
the corresponding position (Fig. S1). These proteins have serine, threonine or glutamine instead and cannot thus substitute for the arginine function. ARHGAP36 is poorly investigated. It has been shown to be involved in Gli transcription factor activation but independent on its GAP domain (98). The ArfGAP and RhoGAP domain-containing Cntd1 (also called ARAP2; Table 1) lacks RhoGAP activity and acts as an Arf6 GAP (99). DEP1 and DEP2 coordinate cell cycle progression and interfere with RhoA and signaling in spite of lacking RhoGAP activity (100). OCRL1 has been shown to interact with GTP-bound Rac1 without stimulation of its hydrolysis (101). p85α and p85β (85 kDa regulatory subunits of the phosphoinositide 3-kinases) can be also included in the list of RhoGAP-like proteins (Table 1; Fig. S1), as they do not show any detectable GAP activity towards different Rho proteins (102). An essential prerequisite of the GAP function is that the GAP domain in order to position its catalytic residue R-282 (p50 numbering) must employ a number of amino acids that are responsible for binding and stabilizing the protein complex (Fig. 6A). Both p85 isoforms lack most of these binding determinants, e.g., Arg-323, Asn-391, Val-394, and Pro-398, along with the conserved amino acids around the arginine finger (p50 numbering; Fig. S3) (4).

Concluding remarks—Unlike the RhoGEF domains (so-called Dbl homology or DH domains), which exhibit high selectivity for the Rho-, Cdc42-, and Rac-like proteins (36), we have found that the RhoGAP domain itself is nonselective and in some cases rather inefficient under cell-free conditions. Thus, we propose that other domains of RhoGAPs confer substrate specificity and fine-tune their catalytic efficiency in cells. They dictate the specificity of the respective RhoGAP most likely through different successive steps: (i) recruitment to a specific subcellular structure at a given time, (ii) release of its (auto)inhibited and most likely membrane-associated state, (iii) recognition and association with the substrate Rho protein, (iv) complementation of an inefficient active site with a catalytic arginine, (v) stimulation of GTPases reaction by orders of magnitudes, and (vi) finally dissociation from the inactivated GDP-bound Rho proteins. One approach of verifying this hypothesis is conducting RhoGAP domain-swapping experiments in cells using two RhoGAPs with verified specificities. Results may show that the specificity of these RhoGAPs remains unchanged irrespective of recombinant RhoGAP domain.

Formation of binary complexes between two classes of proteins, such as Rho proteins and RhoGAPs, is a straightforward biochemical process. However, we have also shown that its detailed description requires a sophisticated approach capable of covering a huge number of combinatorial possibilities incorporated in such molecular system. Our structural analysis based on the interaction matrix aspires to be such an approach. Its application leads to the division of interacting interface into two parts. First of them determines the formation of complexes and supports catalytic mechanism while the second is responsible for the diversity in catalytic activities. Although it remains to be proven whether such approach is also applicable to different protein systems, we believe that its further elaboration will enable a precise prediction of interacting residues in unknown structure of complexes between Rho proteins and RhoGAPs.

A critical issue regarding experimental determination of the specificity of RhoGAPs in cells is that arginine finger mutants, mostly to alanine (RA mutant), are often used to compromise the RhoGAP function. This approach is in principle very useful under cell-free condition but not really optimal in the cells because an RA mutant may provide a similar readout as the wildtype; it interferes with downstream signaling by competing with the effector(s) for binding to the Rho proteins. RhoGAP mutants at this site are able to persistently bind to and sequester the target Rho protein. This most likely displays a similar readout as the activity of wildtype RhoGAP. Instead of the catalytic arginine, we rather recommend mutating critical ‘binding determinants’, particularly Lys-319 and Arg-323 (p50 numbering; Figs. 6 and 7B). Charge reversal of these residues most likely leads to loss of RhoGAP association with its substrate Rho proteins and consequently the activity of the GAP domain. This is not only a tool for determining the specificity of RhoGAPs but also for investigating GAP domain-independent function(s) of the RhoGAPs.
EXPERIMENTAL PROCEDURES

Constructs—Constructs containing GAP domain of human p50 (amino acids or aa 198-439), GRAF1 (aa 383-583), Rich1 (aa 245-499), p190A (aa 1250-1531), OPHN1 (aa 375-583), Abr (aa 559-822), MgcRacGAP (aa 343-620), DLC1 (aa 609-878), DLC2 (aa 644-916), and DLC3 (aa 620-890) were amplified by standard PCR and cloned in either pGEX-4T1 or pGEX-4T1-Ntev vector. All Rho protein constructs have been reported before (35). Human RhoA, its Gln-63 variant to leucine (Q63L mutant) and RhoD were cloned in pRK5-Myc. Human p190 full-length (FL) and its GAP domain, and their Arg-1284 variant to alanine (RA mutant) were cloned in HA-pKH3 and pRK5, respectively. Rat p190A GAP domain (1242-1439) was cloned into pGST-parallel vector (103).

Proteins—All Rho proteins and GAP domains of RhoGAPs were purified as glutathione S-transferase (GST) fusion proteins from Escherichia coli BL21(DE3) pLysS or CodonPlusRIL as described previously (2,104). All Rho proteins and their nucleotide-free forms were prepared as described (2,105).

Fluorescent nucleotides—Various fluorescence reporter groups, including mant, tamra, and cy3 have been coupled to 2’(3’) hydroxyl group of the ribose moiety of the guanine nucleotide GTP via ethylenediamine (EDA) to obtain fluorescent GTP variants (Jena bioscience, Germany) for the analysis of the GAP-stimulated GTP hydrolysis reactions of the Rho proteins.

Kinetics measurements—All GAP-stimulated GTP hydrolysis fluorescence measurements of Rho proteins were performed at 25°C. Fluorescent GTP-bound Rho proteins (premixing 0.3 µM nucleotide-free Rho and 0.2 µM tamra-cy3-labeled GTP) and the catalytic domain of RhoGAPs (10 µM) were rapidly mixed in a buffer containing 30 mM Tris-HCl pH 7.5, 10 mM KH₂PO₄/K₂HPO₄, 10 mM MgCl₂, and 3 mM dithiothreitol using a Hi-Tech Scientific (SF-61) stopped-flow spectrophotometer instrument with mercury xenon light source as described (32). For excitation wavelengths of 546 nm and 550 nm were used for tamra and cy3 fluorophores, respectively, and a 570 nm (tamra and cy3) cut-off-filter (Schott glass) was used to collect emitted light.

Sequence and structural analysis—Sequence alignments were performed with Bioedit program using clustalW algorithm (106). The intermolecular contacts were determined (≤4.0 Å) between RhoGAP and Rho proteins using available Rho-RhoGAP complex structures in the protein data bank (Table S1). A python code has been written by using Biopython modules (pairwise2 and SubsMat.MatrixInfo) (107) to read given PDB and alignment files and returns corresponding interactions pairs in a matrix form. RhoGAP domains discussed in the matrix have sequence similarities between 20-80 % (Table S2) (108) and assumed to have identical fold and form molecular complexes with similar arrangement. All structural representations were generated using PyMol viewer (109).

Structure determination—A mixture of RhoA-GDP and p190-GAP with a small molar excess of RhoA was dialyzed overnight in a buffer, containing 20 mM Hepes, 100 mM NaCl, 5 mM NaF, 5 mM MgCl₂, 5 mM β-mercaptoethanol). The sample was loaded on a Superdex 200 gel filtration column. Fractions containing RhoA-GDP-MgF₃-p190-GAP complex were pooled and concentrated to 8 mg/ml for crystallization trials. The vapor diffusion method was used for crystallization with sitting drops of 1:1 ratio of protein and crystallization reagent. Best crystals grew from JSCG+ screen (Molecular Dimensions) reagent 82. The crystallization conditions were further optimized and a buffer, containing 30% PEG2000 MME, 0.15 M KCSN in 0.1 M MES pH 6.5, produced crystals of diffraction quality. For data collection crystals were frozen in a cryo solution containing mother liquor with an addition of 0.2 M ascorbic acid and 12.5% glycerol. X-ray data were collected at Argonne National Laboratory, South-Eastern Region Collaborative Access Team (SER-CAT) beamline of Advanced Photon Source (Table S3). The structure was solved by molecular replacement method using program Balbes (110) and refined using Phenix (111). Manual rebuilding of the model during refinement was performed using Coot (112). Final refinement statistics can be found in Table S3. Structure was deposited with a PDB accession number 5IRC.
Pull down assay—RhoD and RhoA were pulled down in their activated states as described previously (38). HEK293T cells were seeded in 6 cm dishes. Next day, cells were transfected with 2 µg DNA of pRK5-Myc-RhoA or pRK5-Myc-RhoD together with 1 µg DNA of HA-pKH3-p190 FL (WT) or pRK5-Flag-p190 GAP domain using the Polyplus JetPEI transfection reagent. Cells were incubated 24 h post-transfection, followed by lysis in ice-cold buffer, including 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, and protease inhibitor cocktail (cOmplete™, EDTA-free, Roche). Cell lysates were transferred to pre-chilled tubes and centrifuged at 13,000 rpm for 5 min at 4°C. 1/25 of each cell lysate was transferred to a new tube and 3x SDS-PAGE sample buffer was added. The rest of the sample lysates were transferred to new tubes and GST-fusion proteins on glutathione beads were added. GST-RTKN was added to the pRK5-Myc-RhoA samples, and GST-mDia1 to the pRK5-Myc-RhoD samples. GST-RTKN and GST-mDia1 were overexpressed in E. coli and isolated from the lysate using glutathione beads as described previously (38). Samples were carefully rotated at 4°C for 10 min, then centrifuged and washed four times with 0.5 ml of ice-cold buffer, including 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂. 3x SDS-PAGE sample buffer was added to each sample. Samples were separated on 10% SDS-PAGE gels followed by transfer to nitrocellulose membrane. Proteins were detected with 9E10 mouse monoclonal anti-c-Myc antibody (Covance), 12CA5 mouse monoclonal anti-HA antibody (Roche), and M2 mouse monoclonal anti-Flag antibody (Sigma-Aldrich).
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
M.-R.A. conceived and coordinated the study; E.A., M.J., R.D., K.-T.K., and K.N. designed, performed and analyzed the experiments. K.R., and P.A. performed Pulldown assays; U.D. and A.-V.S. coordinated the structure determination of the RhoA-p190 complex; E.A., R.D., and M.-R.A. designed the study and wrote the paper; all authors reviewed the results and approved the final version of the manuscript.
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PDB DEPOSITION REPORT
The atomic coordinates for “p190A GAP domain complex with RhoA” (Entry Title) submitted by “Derewenda, U., Derewenda, Z.” (Entry Authors) are deposited at the protein database with dataset ID: D_1000219311 and PDB ID: 5IRC.
FIGURE LEGENDS
FIG. 1. Evolutionary conservation of domains of the RhoGAP family.
Domain composition of 66 RhoGAPs is presented according to their phylogenetic categorization based on GAP domain alignment. In addition to a catalytic GAP domain (red) most RhoGAPs have multiple other functional domains, which are probably involved in lipid and membrane binding (blue), protein interaction (green) or enzyme activities (red and orange). A scale of amino acid number in increments of 200 is shown at the bottom; the total number of the amino acids of the respective RhoGAPs is listed in Table 1. Domain properties and statistics are compiled in Table S4 and S5.

Fig. 2 Tamra-GTP and cy3-GTP but not mant-GTP as fluorescent sensors for monitoring GAP-stimulated GTP hydrolysis of Rho proteins in real-time. (A) Chemical structure of fluorescent reporter groups (mant, tamra-EDA and cy3-EDA) coupled to GTP and its non-hydrolyzable analogue GppNHp. (B) Stimulated GTP hydrolysis of Cdc42 and RhoA (0.2 µM) was measured using fluorescent GTP and p50GAP (10 µM) in a Hi-Tech Scientific (SF-61) stopped-flow instrument and a buffer containing 30 mM Tris, pH 7.5, 10 mM K-phosphate, 10 mM MgCl2 and 3 mM dithiothreitol at 25°C as described (2). In contrast to mant-GTP, which did not provide any significant change in fluorescence, cy3-GTP turned out to be most suitable for the Rho isoforms and tamra-GTP most suitable for Cdc42-and Rac-like proteins (Cdc42 is shown as a representative) as well as for RhoD and Rif. Observed rate constants (k_{obs}) for GAP-catalyzed GTPase reactions can be obtained by single exponential fitting of the fluorescence decay using GraFit program.

FIG. 3. Varying activity and broad selectivity of the RhoGAP family proteins.
Individual GTP hydrolysis reaction rates (k_{obs}; values on the bar charts) of 12 Rho proteins (0.2 µM, respectively) in the absence (−) and in the presence of 10 RhoGAPs (10 µM, respectively) are plotted as bar charts. All data shown are an average of 4-5 different experiments. Color coding is the same as in Table 2 and changes from green for very high through yellow for middle and to red for no GAP activity.

FIG. 4. Statistical diagram of the catalytic efficiency of the RhoGAPs.
Values of fold activation are plotted against respective RhoGAP-Rho protein pairs in numeric order. This diagram illustrates the broad spectrum of catalytic efficiencies and substrate-specific properties of various RhoGAPs for the different Rho proteins, which are divided into six efficiency groups as indicated. Color codes are the same as used in Fig. 3 and Table 2.

FIG. 5. Binding affinity of RhoGAPs to Cdc42.
Real-time monitoring of the association reaction rates of GAPs (10 µM) with mantGppNHp-bound Cdc42 (0.2µM) has been measured and represented as bar diagram (left panel) in direct comparison to the reaction rates obtained for the respective GAP-stimulated GTP hydrolysis of Cdc42 (right panel).

FIG. 6. The interaction interface between Rho and RhoGAP proteins.
(A) The interacting residues (<4 Å in distance; color coded) of the Rho-RhoGAP complex are indicated using an open book representation (rotated 90° along a horizontal axis) of the crystal structure of the RhoA-p50 complex (PDB code 1TX4; RhoA: aa 1–181; p50: aa 236–431). Conserved and variable residues are shown in red, blue and black, respectively. Coloring criteria was taken from the interaction matrix in B. (B) Interaction matrix of Rho and RhoGAP proteins. The interacting residues (<4 Å in distance) were determined using the 6 available crystal structures of Rho-RhoGAP proteins complexes counting 8 distinct Rho-GAP pairs (Table S1). They are shown with corresponding residues from the alignment of the GAP and G domains of RhoGAPs and Rho proteins (Figs. S1 and S2) used in this study, respectively. Orientation numbers for interacting residues correspond to the numbering of p50 and RhoA, respectively. Residues sharing more that 60% sequence similarity in alignments (Figs. S1 and S2) are colored: yellow, hydrophobic residues; green, hydrophilic residues; blue, positively charged residues; red, negatively charged residues. Numbers (0–8) in the black-grey-white gradient-colored boxes illustrate the respective contacts found in 8 Rho-GAP pairs. Red and blue boxes represent contacts between conserved and variable regions, respectively.

FIG. 7. Interaction Matrix adapted for the crystal structure of p190 in complex with RhoA.
(A) The crystal structure of the RhoA-p190 complex (PDB code: 5irc) is illustrated in close representation as surface and ribbon (middle panel) and as open book representation rotated 90° along a horizontal axis. The interacting residues (<4 Å in distance) are color coded as in Fig. 6. (B) Overlay of p190, p50 and
Overlay of p190 (PDB code: 5irc), p50 (PDB code: 1TX4) and GAP20 (PDB code: 3MSX) reveals high Structural conservation except for the zoomed variable 1 region. (C) Interaction matrix calculated solely for the RhoA-p190 structure. Corresponding residues from p50 GAP are left as reference. Hotspots are highlighted in agreement with general interaction matrix in Fig. 6. Contacts missing or excessive in the RhoA-p190 structure are shown as red or blue, respectively.

**FIG. 8. p190-FL acts on RhoA in cells but not on RhoD.**
Transiently expressed Myc-tagged RhoD (A, B) and RhoA (C, D) were pulled down from HEK293T cell lysates with Rho-binding domains of mDia1 and RTKN, respectively, as GST-fused proteins in the presence of either HA-tagged p190-FL (A, C) or Flag-tagged p190GAP domain (B, D). R1284A and Q63L variants of p190 and RhoA were used in addition to wildtype (WT). Immunoblots of pulled down samples and the cell lysates were performed using antibodies against the respective tags.
## Table 1 Human RhoGAP family proteins.

All proteins investigated in this study are shown in bold. Proteins analyzed in another studies mostly towards RhoA, Rac1 and/or Cdc42 are underlined. Last eight proteins are GAP-like proteins.

| No. | Entry name | Acc. no. | Gene names or aliases | aa no. |
|-----|------------|----------|-----------------------|--------|
| 1   | 3BP1       | Q9Y3L3   | SH3BP1, ARHGAP43      | 701    |
| 2   | ABR        | Q12979   | AHR, MBB              | 859    |
| 3   | ARHGAP6    | Q43182   | ARHGAP6, RHOGAP6      | 974    |
| 4   | ARHGAP8    | P85298   | ARHGAP8, RGAP1        | 464    |
| 5   | ARHGAP9    | Q9HR9    | ARHGAP9, RGL1         | 750    |
| 6   | ARHGAP11A  | Q6P4F7   | ARHGAP11A, KIAA0015, FAM7B1 | 1023 |
| 7   | ARHGAP11B  | Q9KBH    | ARHGAP11B             | 267    |
| 8   | ARHGAP12   | Q8WV6    | ARHGAP12              | 846    |
| 9   | ARHGAP15   | Q5GC3    | ARHGAP15, BM-024, BM-030, BM-046 | 475 |
| 10  | ARHGAP19   | Q14C8    | ARHGAP19              | 494    |
| 11  | ARHGAP20   | Q9ZP6    | ARHGAP20, KIAA1391, RA-RhoGAP | 1191 |
| 12  | ARHGAP21   | Q15SU3   | ARHGAP21, ARHGAP10, KIAA1424 | 1957 |
| 13  | ARHGAP22   | Q7ZM3    | ARHGAP22, RHOGAP2     | 698    |
| 14  | ARHGAP23   | Q9227    | ARHGAP23, KIAA1301    | 1491   |
| 15  | ARHGAP25   | P42331   | ARHGAP25, KIAA0053    | 645    |
| 16  | ARHGAP28   | Q9ZK2    | ARHGAP28, KIAA1314    | 729    |
| 17  | ARHGAP30   | Q7Z66    | ARHGAP30              | 1101   |
| 18  | ARHGAP39   | Q9C0H    | ARHGAP39, KIAA1468, Virlse, C6GAP | 1083 |
| 19  | ARHGAP40   | Q5TC0    | ARHGAP40, C20orf95    | 622    |
| 20  | ARHGAP42   | A6NE8    | ARHGAP42, GRAF3       | 874    |
| 21  | BCR        | P11274   | BCR, BCR1, D22811     | 1271   |
| 22  | β-chimaerin | P5257    | CHN2, ARHGAP3, BCR    | 468    |
| 23  | CAMGAP1    | Q6ZU4    | ARHGAP27, CAMGAP1, SHD20, PPN05 | 889 |
| 24  | CCGAP      | Q2M123   | ARHGAP31, CDGAP, KIAA1204 | 1444 |
| 25  | Cnt-d1     | Q9684    | ARAP1, CENTD2, KIAA0782 | 1450 |
| 26  | Cnt-d3     | Q8WBN8   | ARAP3, CENTD5         | 1544   |
| 27  | DLC-1      | Q96O1    | DCLC1, ARHGAP7, KIAA1723, STARD12 | 1528 |
| 28  | DLC-2      | Q9V3M8   | STARD13, DCLC3, GT650 | 1113   |
| 29  | DLC-3      | Q92502   | STARD8, DCLC3, KIAA0189 | 1023 |
| 30  | FAM13A     | Q49985   | FAM13A, FAM13A1, KIAA0914 | 1023 |
| 31  | GMIP       | Q9107    | ARHGAP46, GMIP        | 970    |
| 32  | GRAF       | Q91N1A1  | ARHGAP26, GRAF, KIAA0621, OPIN1L | 814 |
| 33  | GRAF2      | A1A4S6   | ARHGAP10, GRAF2, PGAP | 786    |
| 34  | HEMH1      | Q9Z319   | HEMH1, KIAA0023       | 1136   |
| 35  | MacGAP     | Q8N392   | ARHGAP38              | 665    |
| 36  | MacRacGAP  | Q9HHH5   | RACGAP1, KIAA1478, MacRacGAP, CYK4 | 632 |
| 37  | MYO9A      | Q2RT4    | MYO9A, MYR7           | 2548   |
| 38  | MYO9B      | Q13459   | MYO9B, MYR3           | 2157   |
| 39  | N-chimaerin | P388B2   | CHN1, ARHGAP2, CHN    | 459    |
| 40  | OPEN1      | QM08B0   | OPEN1, ARHGAP1        | 802    |
| 41  | PAR1       | Q52L3    | ARHGAP29              | 1261   |
| 42  | p115       | P9H171   | ARHGAP4, KIAA0311, RGC1, RHOGAP4 | 946 |
| 43  | p190-A     | Q9IYV4   | ARHGAP35, RGF1, RGF1F, KIAA1722 | 1499 |
| 44  | p190-B     | Q13017   | ARHGAP5, RHOGAP5      | 1502   |
| 45  | p260       | A91X9    | ARHGAP32, GRB, KIAA0712, RICS | 2087 |
| 46  | p46        | Q97690   | ARHGAP1, CDC242GAP, RHOGAP1 | 439 |
| 47  | p51        | Q8N264   | ARHGAP24, FGFAP       | 748    |
| 48  | RalBP1     | Q15311   | RalBP1, RLIP1, RLIP76 | 655    |
| 49  | RIC1L1      | Q8E8M7   | RACGAP17, RCH1, MTP066, MTP110 | 881 |
| 50  | RIC1L2      | Q179B9   | ARHGAP4, KIAA0672, RCH2 | 818 |
| 51  | SRGAP1      | Q7G67    | SRGAP1, ARHGAP13, KIAA1304 | 1085 |
| 52  | SRGAP2      | Q7M04    | SRGAP2, ARHGAP3, FMBP2, KIAA0456 | 1071 |
| 53  | SRGAP3      | Q6B95    | SRGAP3, ARHGAP14, KIAA0411, MEGAP | 1099 |
| 54  | SYDE1       | Q6ZW3    | SYDE1                  | 735    |
| 55  | SYDE2       | Q5V79    | SYDE2                  | 1194   |
| 56  | TAGAP       | Q9N103   | TAGAP, TAGAP1, FKSG15, ARHGAP7 | 731 |
| 57  | TGGAP       | Q91559   | ARHGAP35, SNX26, TGGAP | 1287   |
| 58  | ARHGAP36    | Q6ZG8    | ARHGAP36              | 547    |
| 59  | Cnt-d1      | Q8WZ64   | ARAP2, CENTD1, KIAA0580 | 1704 |
| 60  | DEP1A       | Q5TB30   | DEPDC1, DEPDC3, DEPDC4 | 811    |
| 61  | DEP1B       | Q9WU9    | DEPDC1, DEPDC8, XTP8 | 529    |
| 62  | FAM13B      | Q9Y3Y3   | FAM13B, FAM13B, FAM13B1 | 915   |
| 63  | INPP5B      | P23019   | INPP5B, OCRL2         | 993    |
| 64  | OCRL1       | Q01968   | OCRL, INPP5, OCRL1    | 901    |
| 65  | P55A        | P27066   | P55B, GRB1            | 724    |
| 66  | P55B        | Q00459   | PIB3, GRB2            | 728    |
Table 2 Catalytic efficiency of RhoGAPs represented as fold activation. The catalytic hydrolysis activities, calculated as fold activation, are divided into six groups according to legend. Fold activation was obtained by dividing the $k_{obs}$ values of GTP hydrolysis reactions by the $k_{obs}$ values of the intrinsic reactions (Fig. 3). Color codes are the same as used in Fig. 3, and correlate in a gradient fashion with green for very high, yellow for middle and red for no GAP activity.

|       | RhoA | RhoB | RhoC | Rac1 | Rac2 | Rac3 | RhoG | Cdc42 | TC10 | TCL | RhoD | Rif |
|-------|------|------|------|------|------|------|------|-------|------|-----|------|-----|
| p50   | 5037 | 11713| 3028 | 5506 | 1571 | 4033 | 2264 | 8116  | 4637 | 3872| 206  | 1093|
| OPNH1 | 104389| 120103| 27163| 9925 | 6106 | 12713| 594  | 31318 | 35036| 9278| 60   | 89  |
| GRAF1 | 26790| 38367| 20771| 4849 | 383 | 825  | 10   | 50108 | 19136| 9568| 255  | 70  |
| p190  | 43671| 11713| 9686 | 6908 | 3826| 7840 | 743  | 6081  | 3217 | 1609| 43663| 89  |
| Rich1 | 20   | 50   | 33   | 1676 | 1424| 240  | 19   | 1732  | 8333 | 50  | 392  | 126 |
| Abr   | 41   | 72   | 446  | 2602 | 930 | 2380 | 75   | 940   | 133  | 67  | 98   | 51  |
| Mgc   | 234  | 380  | 197  | 9756 | 4379| 3674 | 139  | 4780  | 71   | 35  | 8217 | 61  |
| DLC1  | 3731 | 4347 | 1946 | 333  | 35  | 2    | 1    | 306   | 258  | 58  | 40   | 59  |
| DLC2  | 100  | 142  | 102  | 29   | 31  | 1    | 1    | 4     | 27   | 13  | 42   | 52  |
| DLC3  | 83   | 125  | 69   | 19   | 26  | 2    | 1    | 11    | 35   | 17  | 42   | 50  |
Amin et al., Fig. 1

[Diagram of protein interactions with various labels such as DEP1A, DEP1B, ARH GAP08, ARH GAP20, TAGAP, S3P1, RICH1, RICH2, ARH GAP22, p73, ARH GAP25, p115, SrGAP1, SrGAP2, SrGAP3, ARH GAP11A, ARH GAP11B, ABR, BCR, SYDE1, SYDE2, ARH GAP09, ARH GAP12, ARH GAP15, CAM GAP1, ARH GAP21, ARH GAP23, F-chimerin, N-chimerin, p190A, p190B, ARH GAP42, GRAF1, GRAF2, OPN1, Mgc Rac GAP, ARH GAP30, p200, TCGAP, CD GAP, MYO9A, MYO9B, Rap1P, Rho GAP19, Rho GAP26, ARH GAP40, Mac GAP, DLC1, DLC2, DLC3, ARH GAP06, ARH GAP36, Cnt-d2, Cnt-d3, Cnt-d1, FAM13A, FAM13B, PARG1, HMHA1, Gmip, ARH GAP39, P65, P65B, INP5B, OCRL1].

200aa
Amin et al., Fig. 2

A

GTP: $X = O$
GppNHp: $X = NH$

tamra
mant
cy3

B

Cdc12 mantGTP +10μM p50
RhoA-mantGTP

Cdc12 cy3GTP +10μM p50
RhoA-cy3GTP

Cdc12 tamraGTP +10μM p50
RhoA-tamraGTP

relative fluorescence

time (s)

0 0.4 0.8 1.2 1.6 2
0 0.4 0.8 1.2
0 0.4 0.8 1.2
Amin et al., Fig. 4
Amin et al., Fig. 5
Amin et al., Fig. 6

**A**

![Amin et al., Fig. 6 diagram](image)

**B**

| Color Coding of Conserved Residues | GAP Domain Alignment (p50GAP numbering) |
|------------------------------------|----------------------------------------|
| Hydrophobic                        | GAPVLIF                                 |
| Hydrophylic                        | CSTNQMYYW                               |
| Negatively charged                 | DE                                      |
| Positively charged                 | KRH                                     |
| Contact numbers                    | 0                                       |

| Conserved             | Variable 1 | Variable 2 |
|-----------------------|------------|------------|
| G domain alignment    |            |            |
| RhoA numbering        |            |            |
| Conserved             |            |            |
| Variable 1            |            |            |
| Variable 2            |            |            |

**GAP Domain Alignment**

| G domain alignment (RhoA numbering) | Conserved | Variable 1 | Variable 2 |
|-------------------------------------|-----------|------------|------------|
| RhoA numbered                       |           |            |            |
| Conserved                            |           |            |            |
| Variable 1                           |           |            |            |
| Variable 2                           |           |            |            |

**Max. counts**

| Max. counts |
|-------------|
| 4           |
| 8           |
| 8           |
| 1           |
| 3           |
| 8           |
| 5           |
| 8           |
| 7           |
| 3           |
| 8           |
| 5           |
| 3           |
| 8           |
| 2           |
| 4           |
| 4           |
| 6           |
| 5           |

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