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REVIEW

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The neglected terminators: Rho family GAPs in neutrophils

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Abstract

Background: GTPase-activating proteins (GAPs) accelerate the rate of hydrolysis of GTP bound to small GTPases, thereby limiting the prevalence and concentration of the active, GTP-bound form of these proteins. The large number of potential GAPs acting on members of the Rho family of small GTPases raises the question of specificity or redundancy.

Results: In this review, we summarize experimental data obtained on the role of Rho family GAPs in neutrophils, highlight cases where more than one GAP is involved in a physiological function and show examples that GAPs can be involved not only in termination but also in initiation of cellular processes. We demonstrate that the expression-level regulation of GAPs may also occur in short-living cells such as neutrophils. Finally, we provide insight into the existence and structure of molecular complexes in which Rho family GAPs are involved.

Conclusion: GAPs play more complex and varied roles than being simple terminators of cellular processes.

KEYWORDS
GTPase activating proteins, Rho-family small GTPases, neutrophils

1 | INTRODUCTION

GTP-binding proteins are regarded as cellular time switches. In their active, GTP-bound form, they are able to react with specific target proteins but their endogenous GTP-hydrolysing capability limits the duration of the regulated reaction.[1] Timing is determined by the rate of GTP hydrolysis, which is an inherent property of the given protein. In case of small GTPases (smg), the GTP hydrolytic rate is several orders of magnitude slower than the typical value measured for the alpha subunits of heterotrimeric G proteins. Without any further regulation, processes regulated by smgs would be characterized by very poor dynamic properties.[1]

GTPase-activating proteins (GAPs) significantly accelerate GTP hydrolysis by contributing a critical amino acid to the catalytic site.[1] GAPs comprise a core region of approx. 20 kDa where the GTP-binding and hydrolysing capacity resides, surrounded by any number and type of other interacting domains.[1,2] On the basis of the conserved sequence of the GTP-hydrolysing domain, the potential number of GAPs can be estimated from the genome sequence of different species. The number of putative GAPs acting on members of the different subfamilies of smgs is variably high. The situation is especially complex in case of the Rho subfamily, where the potential GAPs outnumber the smgs by a factor of 3 or 4.[3] The large number of the regulatory proteins, their varied domain structure and different substrate specificity raise a long series of questions. This review discusses the complexity of the Rho family of GAPs revealed in neutrophilic granulocytes.

2 | HOW MANY RHO FAMILY GAPS ARE IN A NEUTROPHIL?

In an earlier in silico study, we analysed the distribution of 54 Rho family GAPs in approx. 50 different human tissues on the basis of expressed sequence tag (EST) databases. In addition, we also searched publicly available data of microarray experiments and identified the expressed Rho family GAPs in approx. 20 tissues or cell types.[2] The

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concordant result of the two searches was that in all investigated tissues a large number of Rho family GAPs may be expressed.

For neutrophils, we found two series of microarray data and they indicated expression of approx. 20 different proteins at variable levels. Not surprisingly, the Rho family GAP profile of macrophages was very similar to that of neutrophils, whereas in lymphocytes, almost all investigated proteins seemed to be expressed, several of them at very high intensity.

Experimental data obtained on isolated cells or in genetically modified animals indicate the involvement of at least 11 different Rho family GAPs in various neutrophil functions (Table 1).

3 | HOW MANY RHO FAMILY GAPS ARE INVOLVED IN ORGANIZING A FUNCTION?

Rho family smgs are involved in several vital functions of neutrophils. P67^{phox}, one of the essential subunits of NADPH oxidase, is a direct target of Rac1 and Rac2.[22,23] In this way, the activity of these smgs determines the rate and duration of superoxide production. Direct targets of Rho (mDia), Rac (PAK and WAVE) and Cdc42 (WASP) are involved in regulation of actin polymerization, whereas Rho regulates myosin contractility via its direct target Rho kinase (ROCK).[24] Thus, all three major members of the Rho subfamily of smgs are directly involved in the organization of shape changes, adherence, motility such as chemotaxis or migration through the vessel wall, and phagocytosis, all basic neutrophil functions. In addition, Rho family members are also indirectly involved in the regulation of all these neutrophil functions via their effects on different lipid kinases, for example PI3K and PI4P-5-kinase.[25]

There are a few examples indicating that more than one Rho family GAP may be involved in the regulation of the same molecular process. In an acellular system, Rho family GAPs associated with the cell membrane of human neutrophils have been specifically depleted by antibodies, and changes in the NADPH oxidase activity were followed.[6] Extraction of p50RhoGAP (Cdc42GAP) or ARHGAP25

TABLE 1 Smg specificity and cellular functions of Rho family GAPs investigated in neutrophils

Rho family GAP	Smg specificity	Species	Regulated function	References
ARAP3/CENTD3	RhoA (Arf6)	Mouse	Adhesion, spreading, granule release, chemotaxis	[4]
ARHGAP25	Rac	Mouse	Transendothelial migration	[5]
		Human (cell free)	NADPH oxidase	[6]
		Human/cell culture	Phagocytosis Actin polymerization	[7,8]
ARHGAP1/Cdc42GAP/ p50RhoGAP/RHOGAP1	Rho/Rac/ Cdc42	Human (cell free)	NADPH oxidase	[6,9]
		Mouse	Motility, migration	[10]
ARHGAP35/p190-A RhoGAP/GRLF1	Rac/Rho	Human (cell free)	NADPH oxidase	[6,9,11]
		Mouse	No role in integrin-mediated neutrophil functions and in rheumatoid arthritis	[12]
		Human	β2 integrin activation	[13]
ARHGAP46/GMIP	RhoA	Mouse/human	Azurophilic granule exocytosis.	[14]
ARHGAP15	Rac	Mouse	Migration, NADPH oxidase, phagocytosis, abdominal sepsis, retention in bone marrow	[15,16]
		Human/cell culture	Migration, actin polymerization	[17]
ARHGAP13/srGAP1	Cdc42	Mouse/human	Neutrophil chemotaxis	[18]
BCR/ABR	Rac/Cdc42	Mouse (double knockout)	Acute inflammatory responses	[19]
BCR	Rac/Cdc42	Human (cell free)	NADPH oxidase	[9]
		Mouse	Septic shock NADPH oxidase	[20]
ARHGAP12	Rac	Human/cell culture	Actin polymerization	[8]
ARHGAP43/SH3BP1	Rac	Human/cell culture	Actin polymerization	[8]
ARHGAP21/ ARHGAP10	Cdc42/RhoC	Mouse/human	Hematopoietic stem and progenitor cell development	[21]

Data are based on PubMed search.

resulted in significant increase in superoxide production. Moreover, the two alterations were additive, showing that under resting conditions, both proteins were actively downregulating Rac activity. In contrast, depletion of p190-A (ARHGAP35) did not affect NADPH oxidase activity. The latter finding suggests selectivity in the action of Rho family GAPs.

A more complex situation has been revealed in the phagocytic process. In human neutrophils, we have observed the accumulation of ARHGAP25 around the freshly formed phagosomes.[7] The localization of ARHGAP25 does not overlap with regions enriched in polymerized actin (Figure 1), suggesting that local downregulation of Rac activity impedes actin polymerization. In functional studies downregulation of ARHGAP25 by siRNA in the human model cell line PLB-985 or in primary human macrophages significantly increased, whereas its overexpression in COSphoxFcγR cells prevented, phagocytosis of opsonized yeast particles.[7] The effect depended on the GAP activity of the construct. Interestingly, overexpression of p50RhoGAP, another GAP acting on Rac, did not influence phagocytosis, whereas it prevented the formation of lamellipodia as efficiently as did ARHGAP25. Again, the data point to selective action of GAPs with similar substrate specificity.

A detailed study was carried out subsequently by the group of Sergio Grinstein in macrophages.[8] In a systematic screen, they eventually identified three Rho family GAPs—ARHGAP12, ARHGAP25 and SH3BP1—that showed PIP3-dependent translocation to the forming phagosome, and their downregulation significantly impaired FcγR-mediated phagocytosis of large particles. The fact that none of these GAPs was essential for phagocytosis of small particles indicates different molecular organization of the phagocytic process depending on the size of the particle to be engulfed. Silencing of either of the three critical GAPs on its own was able to drastically reduce phagocytosis of large particles, suggesting a kind of sequential involvement of the different GAPs, possibly in different molecular complexes. These remain to be identified in future.

4 | DO GAPS ONLY TERMINATE SMG-DEPENDENT BIOLOGICAL PROCESSES?

GAPs are regarded in general as terminators of smg-organized cellular processes. In fact, the actual level of active, GTP-bound smg is the result of the balance of GEF and GAP activity—complicated by binding of the GDP-smg to GDI and differences due to the prenylation state of the smg (Figure 2). Genetic depletion or pharmacological inhibition of RacGAPs resulted in enhancement of superoxide production,[15,20,26] suggesting that certain GAPs exert a constitutive activity. Hence, physiological inhibition of a GAP may just as well increase the amount of the active form of the smg as activation of a GEF does. Moreover, simultaneous activation of GEF and inhibition of the corresponding GAP can result in burst-like activation of a smg at a specific location.

In case of neutrophil precursors, there is a nice example in which alteration of a GAP activity initiates an important Rac-dependent biological function. Applying a newly developed quantitative phosphoproteomic analysis, Leo Wang and his colleagues identified ARHGAP25 as a protein that showed highly different phosphorylation profiles in resting and mobilized hematopoietic stem and progenitor cells (HSPC).[27] They could also show that ARHGAP25 activity was essential for mobilization of hematopoietic cells from the bone marrow. Our group revealed that

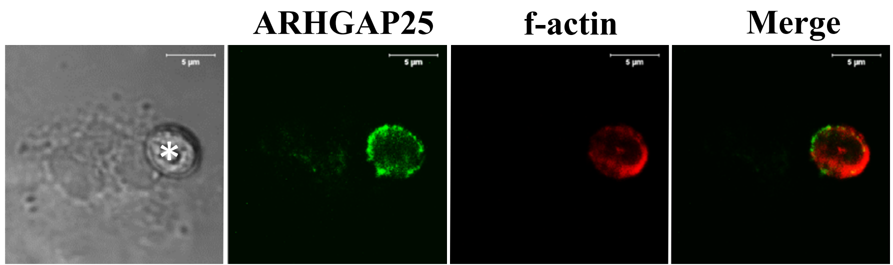

FIGURE 1 Localization of endogenous ARHGAP25 is opposite to filamentary actin around phagosomes. Human neutrophils isolated from peripheral blood[7] were centrifuged to 25 mm in diameter glass coverslips in 10^6/mL concentration. Then, pooled serum-opsonized yeast particles in 10^8/mL concentration were added to the neutrophils and coincubated for 2 min for phagocytosis. Then, cells were fixed with 4% v/v paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 for 5 min and stained with anti-ARHGAP25 polyclonal antibody in 1:1000 dilution for 20 min at RT. Thereafter, staining with Alexa-488-anti-rabbit IgG (in 1:1000 dilution) and in parallel with Alexa-568-Phalloidin (in 1:500 dilution) was carried out for 20 min at RT. Microscope slides were analysed with Zeiss LSM710 confocal microscope, equipped with a 63×/1.40 oil DIC M27 immersion objective. The optical section thickness was approx. 1 μm. Images were processed with Zeiss LSM Image Browser acquisition software. Asterisk shows the phagocytosed yeast particle

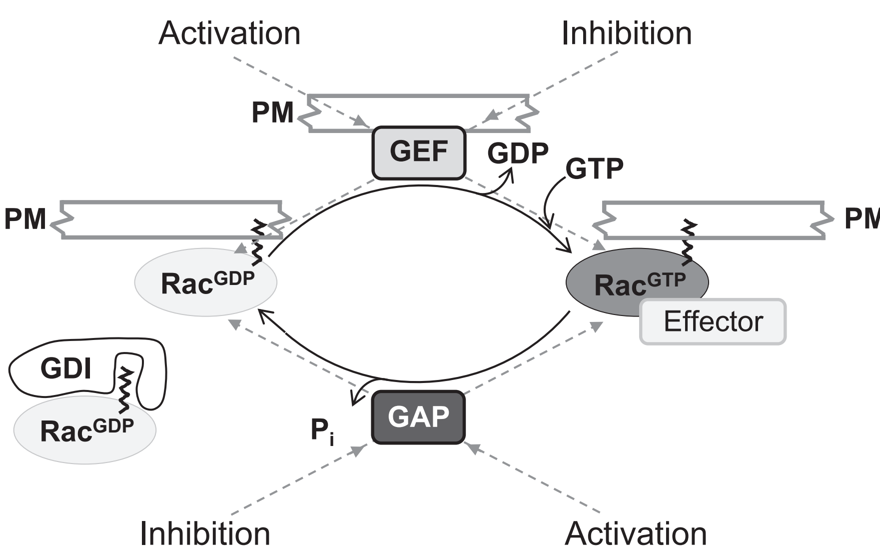

FIGURE 2 The GTPase cycle of smgs. PM: plasma membrane. Dashed lines represent the effect of activation or inhibition of GAPs and GEFs. Zigzag line represents the prenylation of Rho family smgs[3]

phosphorylation of the serine residue at position 363 in ARHGAP25 specifically inhibits the GAP activity and in this way promotes the prevalence of HSPC in the bone marrow. Apparently, dephosphorylation and increased ARHGAP25 activity contribute to cell mobilization from the central hematopoietic organ.

Phosphorylation not only increases or decreases the activity of certain GAPs, but in case of the double-specificity (Rac and Rho) GAP p190-A (ARHGAP35), it also changes the substrate specificity. The N-terminal side of the consensus GAP domain contains a polybasic region that allows electrostatic binding to negatively charged phospholipids. In this state, the protein has mostly RacGAP activity. Within the polybasic region are three PKC phosphorylation sites. Phosphorylation of these serines reverts the phospholipid binding and increases the RhoGAP activity of the protein.[28,29] Opposing alteration of the Rac and RhoGAP activity could be verified also in cellular functions.[30] In contrast, phosphorylation of four amino acids at the C-terminal end of p190-A by GSK decreases both the Rac and the RhoGAP activity of the protein.[31,32] Although the regulatory function could be demonstrated both with human proteins and with murine cells, contribution of the protein to integrin activation differs in human and murine neutrophils.[12,13]

In addition to phosphorylation, GAP activity can be altered in numerous other ways.[33] Neutrophils are characterized by a short lifetime (see Hellebrekers et al., in this issue); hence, expression-level regulation is not what one would typically expect. Nevertheless, our preliminary results indicate that the amount of mRNA coding for ARHGAP25 decreases following exposure to opsonized bacteria, whereas in control cells, no significant change is observed (Figure 3A,B). Detectable decrease in the protein level accompanies the reduction of the coding mRNA (Figure 3C,D), possibly contributing to the enhancement of Rac activity under conditions in which increased cytoskeletal rearrangement is needed.

5 | ARE GAPS LONELY PLAYERS?

GAPs acting on Rho family smgs are present in every part of the neutrophil. In cell fractionation assays, their effect could be shown both in the membranous and in the cytosolic fractions.[9] In microscopic studies, we see evidence of translocation of GAPs and selective association with the plasma or intracellular membranes (as shown for ARHGAP25 above). However, in case of neutrophils, our knowledge is poor on specific molecular complexes composed of one or another Rho family GAP, although the diverse domain structure of the proteins and examples from other cell types predict a highly regulated molecular organization.

One of the important but undecided questions is the following: What exactly do Rho family GAPs react with, the free, uncomplexed smgGTP or smgGTP in complex with its target molecule? Formulated in another way: Does GAP really terminate a smgGTP-dependent reaction or only prevents the reactivation? Structural data obtained by X-ray diffraction are only available for the GAP domain of a few Rho family GAP proteins, whereas folding and steric structure of the numerous other domains are unknown. Susan Smith applied a modelling approach to get insight into this problem. Using the known structure of the complexes of Rac with its target protein p67^{phox} and of Rac/Rho with the GAP domain of several GAPs, she constructed a model of the trimolecular complex. According to her analysis, the Rac-p67^{phox} complex allows room to accommodate the GAP domain; thus, a tripartite arrangement could be

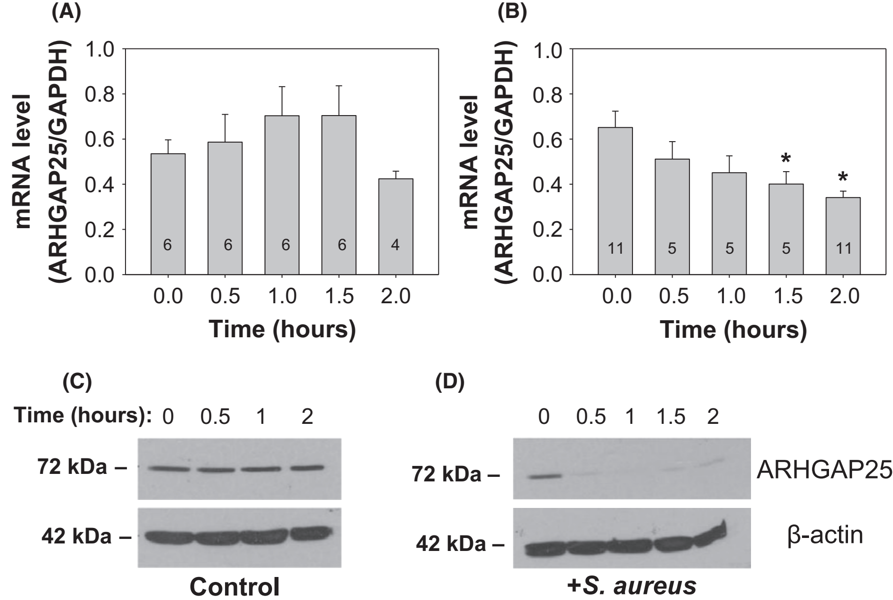

FIGURE 3 Bacterial stimulus results in a reduced mRNA and protein level of ARHGAP25 in human neutrophils. Human neutrophils were isolated from peripheral blood. Then, cells were stimulated with pooled serum-opsonized *S. aureus* for the indicated time. Opsonization of the bacteria and details of stimulation are described in.[40] Total mRNA was isolated from the cells, and cDNA was prepared. Then, real-time PCR was carried out according to the protocol detailed in,[41] with ARHGAP25-specific primers and probe, and the cDNA as template. Panel A, shows the results of control experiments, in which cells were treated only with serum, but without bacteria. Panel B, shows the results of experiments, in which neutrophils were stimulated with opsonized bacteria. The number of independent experiments is given in the columns. Bars represent the mean + SEM of the indicated independent experimental results. *: $P<0.05$. Panels C, D, ARHGAP25 protein amount is decreased in human neutrophils treated with opsonized bacteria. Cells were stimulated with pooled serum-opsonized *S. aureus* for the indicated time, as described above. Then, cells were lysed, and SDS-polyacrylamide gel electrophoresis and Western blot were carried out as described in.[7] Western blot was treated with anti-ARHGAP25 polyclonal antibody in 1:1000 dilution and with anti-β-actin antibody in 1:10 000 dilution

possible.[6] This model suggests that the GAP could have access to RacGTP also in complex with its target protein—at least with p67^{phox}—and really terminate the Rac-regulated process. Experimental data showing an increase in superoxide production upon inhibition of GAP action after the assembly of the NADPH oxidase complex has occurred are in agreement with the suggestions of this model.[34]

Moreover, the steric model of the tripartite complex revealed an interface between the target protein p67^{phox} and the GAP protein,[6] which could serve as selectivity filter for the interacting Rho family GAP. This situation would then be similar to the tripartite structures described for smg-target protein-GEF interactions.[35,36]

A few interesting examples in other cell types have shown that the supramolecular organization of GAPs is essential for their function. In case of epithelial junctions, the term "zonular signalosome" has been introduced to summarize the complex consisting of zonular proteins and different RhoGEFs and RhoGAPs that assure the anchoring of the junction as well as its contacts with the cytoskeleton.[37] More direct data are available in neural studies. Synaptogenesis of hippocampal neurons was shown to depend both on the RacGEF Tiam1 and on the RacGAP Bcr. A direct molecular interaction was revealed between these two proteins that were essential in limiting Rac1 activation. Furthermore, proper development of synapses initiated by EphB receptor critically depended on the prevalence of and interaction between RacGEF and RacGAP in the complex.[38] Finally, in a very recent study, a glimpse is offered into the dynamics of local changes of Rho and Rho-target activity via temporal shift of recruitment of GEF-H1 (ARHGEF2) and the RhoGAP Myo9b.[39]

6 | CONCLUSION

Although the verified enzymatic activity of GAPs consists of acceleration of the GTP hydrolytic rate and thereby attenuation of the biological function of the relevant smg, these proteins play multifaceted roles in organization of various cellular functions. Their contribution thus extends far beyond the simple role of a "terminator," and occasionally, they may even function as "initiators."

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CONFLICT OF INTEREST

The authors have no financial conflicts of interest.

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